IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Henri Arnold De Bruyn et al.	**Examiner:**	Magali P. Theodore
Serial No.:	10/501,356	**Art Unit:**	1791
Filed:	February 11, 2005	**Docket:**	18005
For:	BINDER COMPOSITION AND METHOD FOR TREATING PARTICULATE MATERIAL		

Confirmation No.: 7585

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF HENRI A. DE BRUYN

Sir:

I, Henri A. de Bruyn, declare and state as follows:

1. I am a co-inventor of the above-identified application and I have complete knowledge of all aspects of the invention.

2. I have been the chief executive of companies researching this field for twelve years. In this time I have directed research programs to investigate numerous phenomena of a chemical or engineering nature. I completed a Bachelor of Commerce degree and received a Master of Business Administration (MBA) degree from the University of Pretoria in 1967. I have been working with binder compositions for more than fifteen years, and have written 263 technical bulletins as publications thereon. To further illustrate my expertise in the field, for the convenience of the United States Patent and Trademark Office ("USPTO"), I have attached herewith a copy of my Curriculum Vitae as Exhibit A.

3. I have been requested by counsel to review the Official Action dated July 30, 2009 ("Office Action"), and in particular, to comment upon the distinction between humic acid and fulvic acid, and the reasons why fulvic acid provides an advantage over humic acid as a component of the claimed binder (or matrix) composition.

4. Fulvic acid is that fraction of humic substance (i.e., humins) that is water-soluble (at nearly all pH conditions) and remains in solution after removal of humic acid by acidification. In particular, fulvic acid is highly water-soluble in acidic, neutral, or alkaline conditions, whereas humic acids are water-insoluble in acidic or neutral conditions and water-soluble only in alkaline conditions. For support of these concepts, reference is made to the reference Michael E. Essington, Soil and Water Chemistry: An Integrative Approach, Chapter 4.4 "Humic Substances" (pp. 155-181), CRC Press LLC, Boca Raton, FL (c) 2004 (ISBN: 0-8493-1258-2), as attached herein as Exhibit B. Therein, the differences between humic and fulvic acids is explained in detail.

5. Since the binder is often utilized under acidic conditions, fulvic acids provide the advantage of being completely soluble under these conditions. Some of the advantages of the higher solubility of fulvic acids are discussed below.

6. A particular advantage of the greater water solubility of fulvic acid is that, during mixing of the fulvic acids with other components of a matrix material, the greater water solubility permits the fulvic acid to disperse throughout the matrix material in a highly uniform manner so as to provide an even and consistent strength throughout the matrix material. In contrast, humic substances other than fulvic acids (e.g., humic acids and humins) tend to form suspensions during mixing with the components of the matrix material. These suspensions quickly destabilize to form precipitates of the humic

substances, and this can cause problems during storage and dispensing operations. Furthermore, the precipitation of humic substances during mixing in acidic conditions results in a matrix material with a much less even and consistent strength.

7. Humic acids are complex with various structures called pseudo structures. Reference is made to the paper P. McCarthy, et al., "The Principles of Humic Substances," *Soil Science*, vol. 166, no. 11, pp. 738-751 (2001), attached herein as Exhibit C. These moieties are coupled by weak bridges but are easily solvated (surrounded by water), thereby making the material hydrophilic. This is an unwanted property.

8. The structure of humic acids can be visualized as a sphere with a hydrophilic exterior and a hydrophobic interior. In acidic conditions, humic substances tend to contract due to their flexible nature. Contraction is an unwanted property in building and construction materials (e.g., road materials) as it produces small voids that allow the ingress of water, and this softens the material, reduces the compacted strength of the material, and predisposes the material to cracking. In contrast, fulvic acid has hydrophobic characteristics (i.e., by a predominance of aliphatic groups), which makes fulvic acid more resistant to infiltration by water, thereby helping to keep water out of the material and maintaining integrity of the material. Furthermore, due to the smaller size of the fulvic acid molecule, any contraction of fulvic acid molecules is significantly less than for the larger humic acid molecules.

9. Fulvic acid molecules are also structurally labile and contain an abundance of polar and ionizable functional moieties, which accounts for their solubility in both acidic and alkaline solutions. These concepts find support in Exhibit B. The abundance

of functional moieties also advantageously enables fulvic acid to form bonds with the polymer as well as with the molecules of the soil or other material the binder is stabilizing. The bonding provided by fulvic acid imparts greater strength to the material (and thus, a more durable material), whereas humic acid, which is less functionalized, engages in fewer and weaker bonds, thereby imparting less strength to the material.

10. Furthermore, the high concentration of carboxyl groups in fulvic acids provide the additional advantage of functioning as a source of hydrogen ions that are continually released into the matrix material during processing. The released hydrogen ions help polymerize the binder, thereby increasing the crosslinking and strength of the matrix material. This characteristic of fulvic acids is so pronounced that polymerization of the binder can be effected solely by the fulvic acid, even if no other acid is added. In contrast, humic acid possesses a significantly lower concentration of carboxyl groups, and thus, humic acid does not exhibit similar beneficial effects when added into a matrix material.

11. Fulvic acids also have significantly smaller molecular weights compared to other humic substances, such as humic acid. Humic acids display a continuum of molecular masses ranging from approximately 1000 Da (Daltons) to 500,000 Da, with a mean of about 50,000 Da. More typically, fulvic acids have a limited range of molecular mass varying from around 300 to 2,000 Da, with a mean of about 960 Da. Reference is made to Essington (Exhibit B), and also to R. S. Cameron et al., "Molecular Weight and Shape of Humic Acid from Sedimentation and Diffusion Measurements on Fractionated Extracts," *Journal of Soil Science* vol. 23, no. 4, pp. 394-408, (1972), attached herein as Exhibit D. The smaller size of fulvic acid molecules allow these molecules to more

effectively spread when combined with water into the soil, where the fulvic acids can combine with other humic substances and the binder components, to form larger macromolecules during setting. The formation of larger macromolecules results in an improved crosslinking of polymer chains and substrate. The improved crosslinking results in an increased binder strength and water resistance as compared to matrix materials that use humic substances other than fulvic acid.

12. Fulvic acids are also significantly more effective crosslinking substances (i.e., by weight) than other humic substances. Therefore, minute quantities of fulvic acid can be mixed with the other components of the matrix material to achieve strength improvements significantly greater than an equivalent amount of humic substances not containing fulvic acids. Furthermore, by the superior ability of fulvic acids to impart structural integrity and other improved characteristics to a matrix material, use of fulvic acids expands the range of usable soils by including those soils that would otherwise not be usable, even with the addition of humic acids, because of their poor physical characteristics.

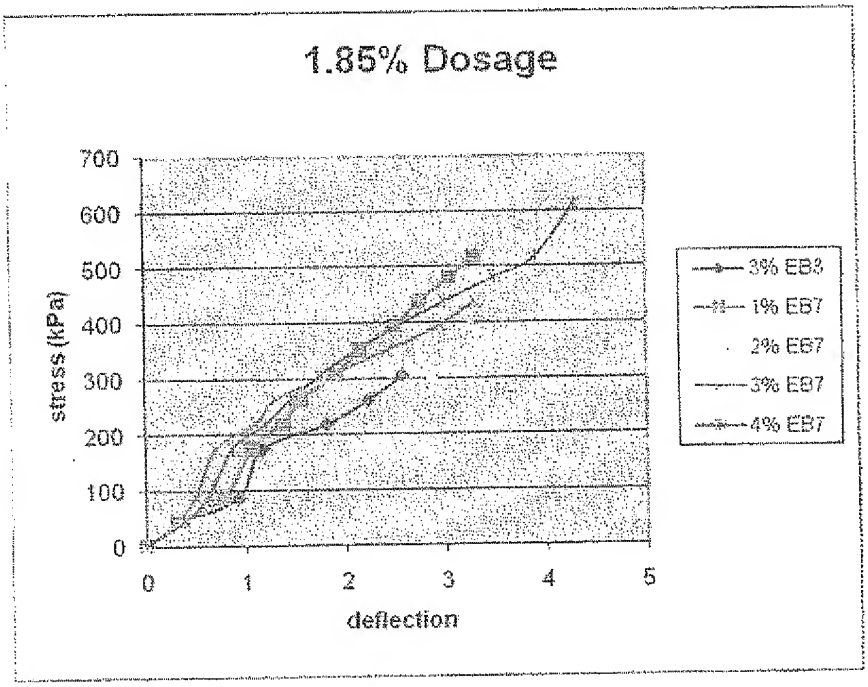
13. Furthermore, synthetic forms of fulvic acid can be manufactured by more straightforward and facile methods than the manufacture of humic acids. Fulvic acid can be manufactured by the oxidation of readily available plant sugars and the addition of some naturally occurring enzymes. Formation of a synthetic fulvic acid can be accomplished within a few weeks. In contrast, the formation of humic acid usually takes place in the fermentation process of a variety of plant material over a period of many months or years. To accelerate this process, the fermenting conditions are controlled by

processes that significantly increase capital and running costs. Accordingly, fulvic acid is not only advantageous by its superior properties, but also more commercially feasible.

14. In further support of the above assertions, I herein submit a graph, as shown below, which evidences an improvement in the incorporation of fulvic acids, in place of humic acids, in a matrix material of the invention. The graph illustrates a significant increase in strength (40% to 100%) of the binder / soil matrix (average dosage 2.5%) when fulvic acid is added. A relatively pure form of fulvic acid was used in this test. The binder (3% of soil weight) containing 27% Urea, 70% UFC and 3% (EB3) modified citric acid (of 1.5% of soil weight) together with an equal (1.5%) amount of anionic bitumen emulsion was mixed into enough water to attain optimum moisture content into a ferricrete soil. The mixture was then compacted and dried at 50°C for 48 hours in an oven with a fan. The EB3 citric acid was replaced with fulvic acid (EB7) in 1%, 2%, 3% and 4% dosages in the next four samples (similarly prepared and dried). The unconfined compressive strength (UCS) was determined after 80 minutes of soaking under water (equivalent to four hours of soaking for larger samples). The deflection (in mm) was then measured.

15. The attached graph shows the correlation between applied kPa stress and deflection. The 1% fulvic acid sample (compared to 3% citric Acid with no fulvic acid) reached 3.5 mm deflection at 500 kPa compared to < 3mm and 300 kPa unconfined compressive strength when the samples failed under the load. Similarly, the 2% fulvic acid sample was able to handle > 4 mm deflection and failed at 480kPa, which is significantly stronger than the sample with no fulvic acid. It is noteworthy that the 3% and 4% fulvic acid samples were also much stronger than the sample with no fulvic acid.

Significantly, the 4% fulvic acid sample was 100% stronger than the sample with no fulvic acid at 600kPa.



16. Reference is also made to Figure 9 of the application as filed, and page 40, lines 13-16 of the application as filed. These portions of the application also show the improvement in strength when fulvic acid is added to soil containing organics.

17. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 29 Dec 2009

Henri A. de Bruyn

EXHIBIT A

CV Summary of Henri Arnold de Bruyn, inventor of the UF resin binder described in US Patent Application No. 10/501,356.

1. Academic background:

   - Completed B.Comm and MBA at Pretoria University (1960's)
   - Studied several years for Doctorate (DBA) (not completed as study fields became too wide for one thesis)

2. Has written (some confidential):

   - 263 Technical Bulletins related to soil binder and its application
   - Roads Design Manual
   - Roads Construction Manual
   - Logistics Manual

   (Not published in scientific journals).

3. Has interviewed several hundred engineers, mostly civil but also chemical and other and worked intensively for 15 years in soil stabilization field.

4. Has worked closely with Dr. Gerhard Overbeek (doctor of Chemistry) and Frans de Bruyn (B.Sc. Pr. Ing. MBA) in R&D of the binder.

5. Has conducted / caused / analysed more than 3 000 laboratory tests done with the binder and variations and various formulations, ratios, soils compaction ratio, moisture contents etc.

6. Has tested dozens of variations of field applications in divergent circumstances.

7. Has managed or performed executive functions in various companies since 1961.

EXHIBIT B

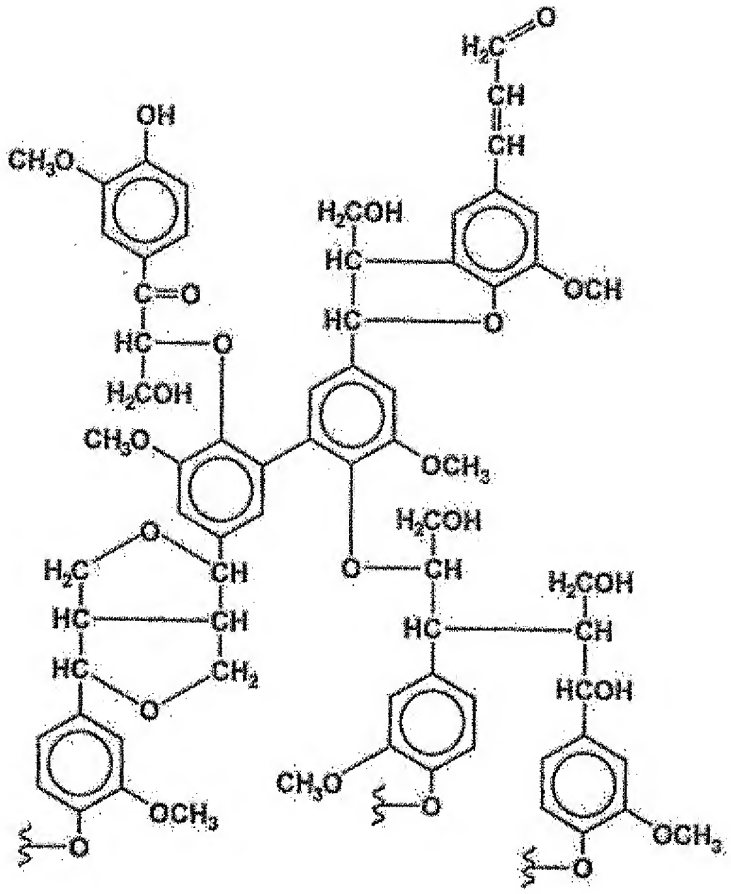


FIGURE 4.20 A possible structural configuration of lignin.

4.4 HUMIC SUBSTANCES

As classically defined, humic substances are (Stevenson, 1994):

> A series of relatively high-molecular-weight, brown- to black-colored substances formed by secondary synthesis reactions; the term is used as a generic name to describe the colored material or its fractions obtained on the basis of solubility characteristics; these materials are distinctive to the soil (or sediment) environment in that they are dissimilar to the biopolymers of microorganisms and higher plants (including lignin).

Although this definition is commonly cited, it provides very little information about the chemical nature, properties, and environmental role of humic substances. Indeed, the definition is vague relative to the genesis and function of humic substances in soil, instead focusing on color characteristics and the solubility characteristics of its various fractions. However, it clearly sets forth the long-held view that humic substances are macromolecular (high-molecular-mass), formed naturally (and indeed a substance that is unique to soil), and structurally similar to microbial and plant biopolymers (like lignin and other phenylpropanoids), but derived by the re-polymerization of the byproducts of biopolymer decomposition (secondary synthesis).

A more recent definition of humic substances (after MacCarthy, 2001) offers insight into their chemical nature and origin:

Humic substances comprise an extraordinarily complex, amorphous mixture of highly heterogeneous, chemically reactive yet refractory molecules that serve a key role in the Earth's ecological system, produced during early diagenesis in the decay of biomatter, and formed ubiquitously in the environment via processes involving chemical reaction of species randomly chosen from a pool of diverse molecules and through random chemical alteration of precursor molecules.

The characteristics of humic substances that are described in this definition are discussed below. It cannot be overstated that humic substances are exceedingly complex, heterogeneous, and reactive components of the soil environment that are relatively resistant to microbial decomposition. Further, and seemingly contradictory to the definition of Stevenson (1994), the definition of MacCarthy (2001) makes no mention of the perception that humic substances are macromolecular. This view has been hotly debated by humic scientists and there is no consensus as to whether humic substances are (1) large macromolecules formed by biotic and abiotic synthesis reactions that repolymerize the products of biomolecule decomposition, or (2) supramolecular associations of heterogeneous and relatively small molecules that are derived from the decomposition of biomolecules.

Humic substances are partitioned into three main fractions: humic acid, fulvic acid, and humin (Figure 4.1). These classes of humic substances are operationally defined by their differential aqueous solubilities in acidic and alkaline solutions, not by their innate structural or chemical characteristics. One method for isolating the fractions of humic substances from soil is illustrated in Figure 4.21 (after Swift, 1996). The extraction of soil humus or acid-washed soil with a 0.5 *M*

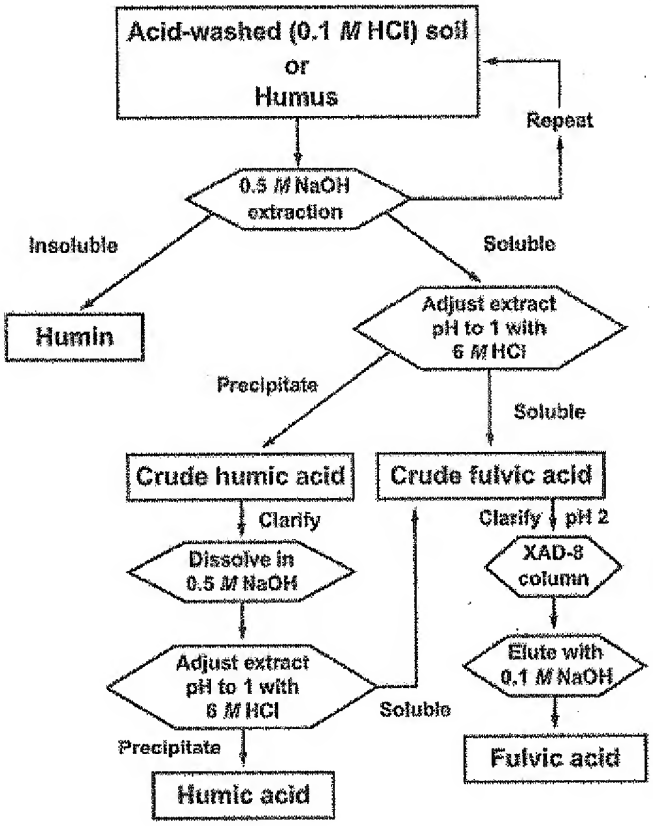


FIGURE 4.21 A fractionation scheme commonly employed to isolate the humic substances (after Swift, 1996). Note that the three separates (humin, humic acid, and fulvic acid) are strictly defined by their differential solubilities in acidic and alkaline solutions.

NaOH solution solubilizes the humic and fulvic acid fractions of soil humus. The organic component that is not solubilized after repeated alkali extraction of soil humus is defined as the humin fraction. The alkaline extract is then treated with concentrated (6 *M*) HCl in order to adjust the pH to approximately 1. The precipitate that forms as a result of the pH adjustment is the crude humic acid fraction, while the organic material remaining in solution is the crude fulvic acid fraction. Both these fractions may be clarified to (1) separate nonhumic substances and fulvic acid from the crude humic acid precipitate, and to (2) separate nonhumic substances from the soluble crude fulvic acid fraction. In the case of the former, the crude humic acid fraction is first redissolved in 0.5 *M* NaOH, and then reprecipitated by acidification with 6 *M* HCl. The resulting precipitate is called generic humic acid. The acid solution is combined with the solution containing the crude fulvic acid fraction. The separation of nonhumic substances from fulvic acid is performed by passing the acidic crude fulvic acid solution through an XAD-8 resin column (or equivalent). The XAD-8 resin is a nonionic, macroporous (25-μm pore size), methyl methacrylate ester polymer. In acidic eluents, the acidic functional groups of fulvic acid are protonated and adsorbed by the resin, while inorganic solutes and nonhumic substances (such as polysaccharides and low-molecular-mass organic acids) pass through the column. The fulvic acid fraction is then removed from the resin by eluting with an alkaline solution, yielding generic fulvic acid.

4.4.1 Genesis of Humic Substances

Several mechanisms, or pathways, have been proposed to describe the genesis of humic substances. The five principal and credible mechanisms are illustrated in Figure 4.22. There are essentially two categories of pathways: (1) those that are purely biological and involve the enzymatic decomposition of biopolymers and the enzymatic recombination of the microbial byproducts (pathways 2, 3, and 4),

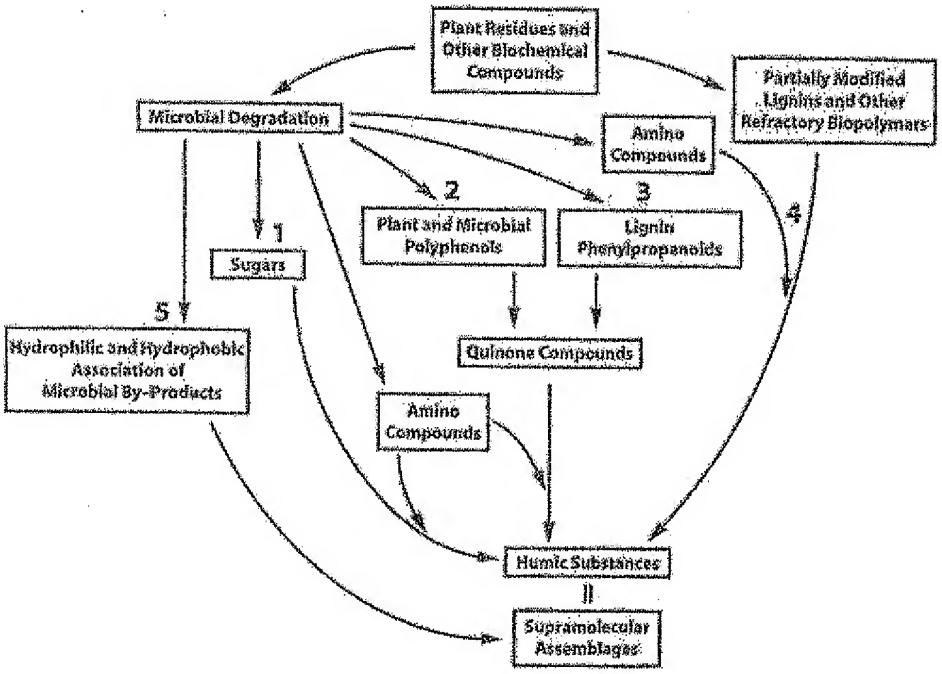


FIGURE 4.22 Numerous pathways have been proposed to describe the genesis of humic substances. The plausible pathways, either producing humic substances alone or in combination, are (1) sugar-amine theory; (2) polyphenol theory; (3) and (4) lignin-protein theory; and (5) self-aggregation theory.

and (2) those that involve the biotic decomposition of biopolymers and the abiotic assemblage of macromolecular structures (pathway 1) or aggregates (pathway 5). One of the oldest concepts of humic substance formation is the sugar-amine theory (pathway 1). In this pathway, monosaccharides and amino compounds produced during microbial metabolism of nonhumic substances recombine via purely abiotic condensation reactions (water is released) and result in the formation of multiple and principally covalent bonds. The products of these reactions are brown nitrogenous polymers, or melanoidins, that contain furan, pyrrole, pyridine, and other heterocyclic aromatic residues. A process that is commonly cited to explain abiotic condensation in soils is the Maillard reaction: a random condensation of amino acids and monosaccharides that results in the formation of complex macromolecular structures of varying size and solubility. Laboratory-formed melanoidins are similar to natural humic substances, particularly with respect to their high degree of cross-linkage, insolubility in water, and nonhydrolyzability. The Maillard reaction is highly sensitive to reaction conditions, being favored in alkaline systems that contain large amounts of monosaccharides, proteins, peptides, and amino acids. However, as discussed by Burdon (2001), alkaline soils do not contain greater quantities of humic substances than acidic soils, and the concentrations of free sugars and amino acids in soils are exceedingly low. Further, the products of the Maillard reaction contain primarily heterocyclic N, rather than the amide N common to humic substances.

Two of the pathways illustrated in Figure 4.22 (3 and 4) are actually subpathways described by the lignin-protein theory, or simply the lignin theory. Both of these pathways view lignin as the source of humic substances (even though humic substances form in soils that lack lignin input from higher plants). In pathway 4, plant lignins are only partially (superficially) modified by soil microbes, such that the polymeric structure of the macromolecules essentially remains intact. Pathway 3 builds on pathway 4 by allowing for the enzymatic cleavage of the β-*O*-4 bonds in the lignin macromolecule and the generation of lignin and dilignol polypropanoid units (Figure 4.19). Irrespective of the degree of lignin decomposition, the modification that is required for the repolymerization of the lignin monomers and larger lignin structures is the demethylation of the phenolic ester leading to a catechol (*p*-benzenediol, Figure 4.23). In addition, the oxidation of the propanol side-chains results in the formation of carboxyl groups. Carboxyl groups may also result from the cleavage of the aromatic rings in lignin. The catechol is enzymatically oxidized further to the *o*-quinone (by polyphenoloxidases), which undergoes condensation with amino compounds and other quinones. The amine-quinone condensation reactions illustrated in Figure 4.23 result in the production of an aromatic amine, which will undergo additional condensation with other quinones to form a polymeric humic substance. The macromolecules that initially form in pathways 3 and 4 tend to be relatively oxygen-poor, and relatively insoluble in the alkaline solutions employed to operationally describe humic and fulvic acids, thus, these substances would be defined as humin. As the oxidation of these macromolecules continues, they become more enriched in oxygen-bearing functional groups (carboxyl, carbonyl, phenolic-OH). As a result, they display greater solubility in alkaline media and insoluble in acidic solutions; thus, they are operationally defined as humic acids. With continued oxidation, these substances would ultimately develop into both acid- and base-soluble compounds—the fulvic acids. Therefore, lignin theory describes the least humified substances as humins, and the most humified substances as fulvic acids.

Perhaps the most widely accepted theory of humic substance formation is the polyphenol theory (pathway 2). Unlike the lignin theory, the polyphenol theory states that the quinones originate from both plant and microbial sources. This theory also considers all biopolymers to be decomposed to their monomeric units before enzymatic repolymerization occurs. By this mechanism, the complexity of humic substances increases with age, with the formation of fulvic acids occurring initially. The continued incorporation of monomeric decay products into humic macromolecules ultimately leads to the most humified substance, humin. This sequence of humification is counter to that proposed in the lignin theory.

The polyphenol theory is diagrammed in Figure 4.24. Lignin is still considered to be the principal source for phenolic substances, although these substances may also be derived from

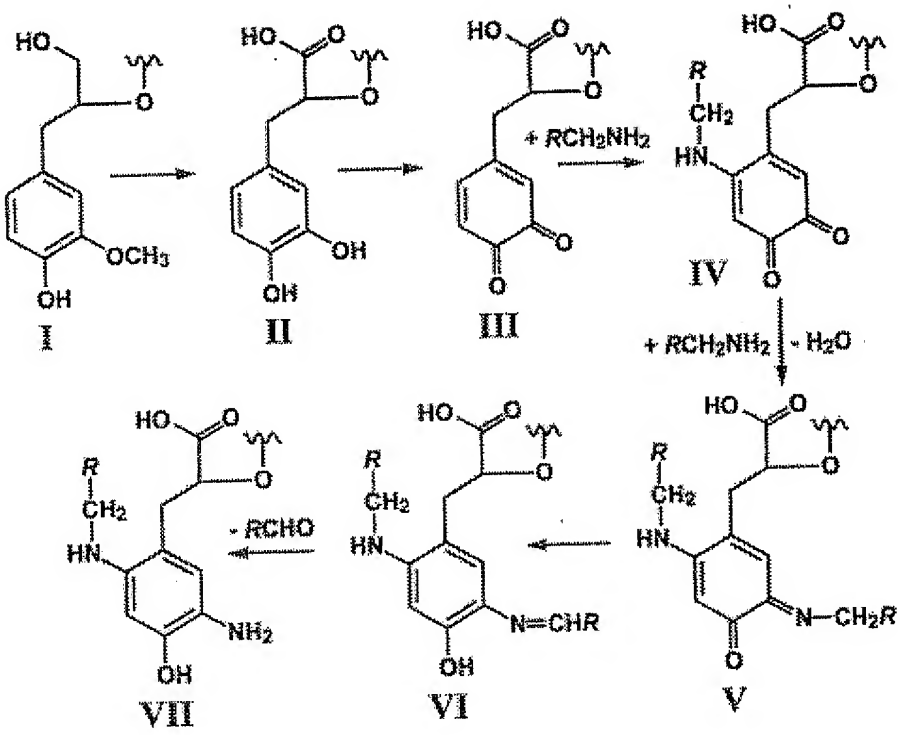


FIGURE 4.23 The polymerization of phenylpropanoid units is theorized to occur through a series of microbially mediated processes as illustrated for the guaiacyl unit (I). Demethoxylation and oxidation of the propanoid result in a catechol (II), which is then oxidized to the *o*-quinone by polyphenoloxidases (III). Amino acids and other amino compounds (amino sugars) react with the quinone to produce the substituted quinone

glycosides that contain aromatic structures (e.g., antibiotics), tannins (which contain flavonoids and phloroglucinol, Figure 4.17), aromatic amino acids (e.g., phenylalanine and tyrosine, Figure 4.10), and microbial synthates. Once produced, the phenolic substances are oxidized to quinones by polyphenoloxidases. Self-polymerization of quinones may occur in soils and has been shown to occur in laboratory cultures. However, the condensation of quinones is greatly enhanced in the presence of amino compounds (Figure 4.23), such as amino sugars and amino acids, and results in a product that has many of the characteristics of humic substances.

In the pathways described above (1 through 4), humic substances are presumed to be comprised of macromolecular, polymeric structures. These substances result from condensation reactions that form numerous cross-linkages that involve strongly covalent bonds, such as the C—C and C—N bonds. However, there is an alternate paradigm to the traditional view of humic substances as macromolecules. This view is the basis for pathway 5 (self-aggregation theory), which considers humic substances to be supramolecular associations of relatively small molecules that have self-organized into relatively large molecular entities. The small molecules in these suprastructures consist of plant and microbial residues and their microbial degradation products. During the enzymatic and oxidative depolymerization of plant biopolymers, such as lignin (shown in Figure 4.20), tannins, and cutins, carboxyl groups are formed. Some portions of these monomeric or larger units may remain relatively unaltered and relatively hydrophobic. The unaltered portion of the unit

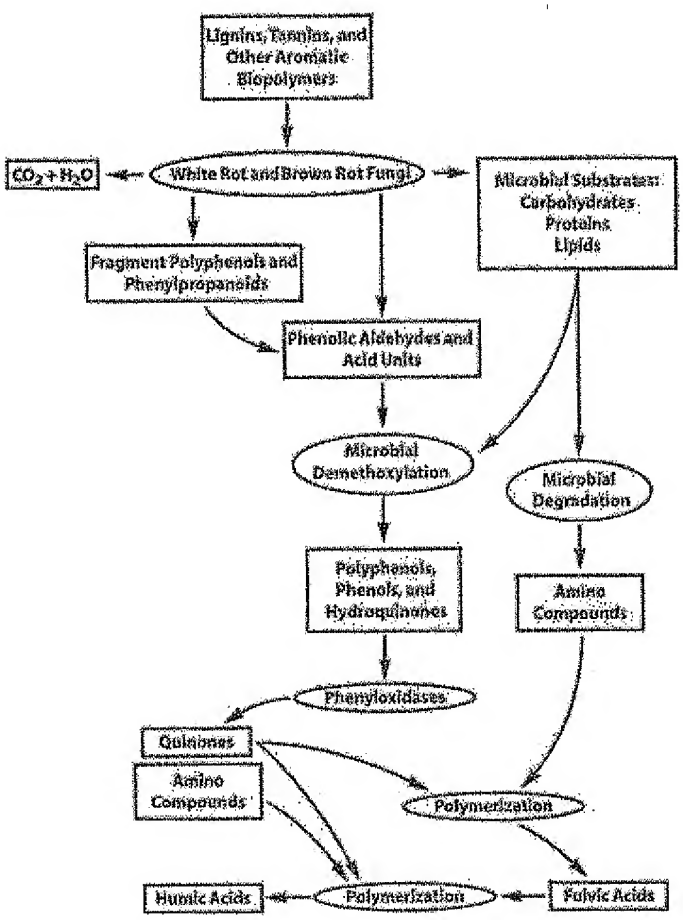


FIGURE 4.24 The polyphenol theory of humic substance production differs from other theories in that the phenolic building blocks may be derived from biopolymers other than lignin. The individual phenolic units are then polymerized according to the scheme shown in Figure 4.23.

will contain aromatic and aliphatic structures and will be relatively nonpolar or hydrophobic; whereas, that portion of the molecule containing the carboxyl group will be polar or hydrophilic, if not ionic (Figure 4.25a). In essence, the depolymerization of biomolecules may result in substances that are amphophilic and surfactant-like. These amphophiles may aggregate on mineral surfaces or in solution. At low concentrations in soil solutions, the amphophilic units remain relatively dispersed. However, as solution concentrations increase above some critical concentration, the units aggregate to form a micelle. The critical concentration is called the critical micelle concentration. A micelle is a globular unit composed of several amphophiles arranged such that the hydrophobic portions of the molecules are in the interior and the hydrophilic portions are on the exterior (Figure 4.25b). These micelles are stabilized by π-π bonds and weak forces that do not contain covalent bonds. These weak forces include attractive hydrophobic interactions, such as van der Waals forces, and hydrogen bonds (described in Chapter 2). The solubility of the micelle units is dictated by the number and types of acidic functional groups that make up the exterior of the micelles. If the units contain a large number of acidic functional groups that are deprotonated, the units will remain soluble as part of the fulvic acid fraction.

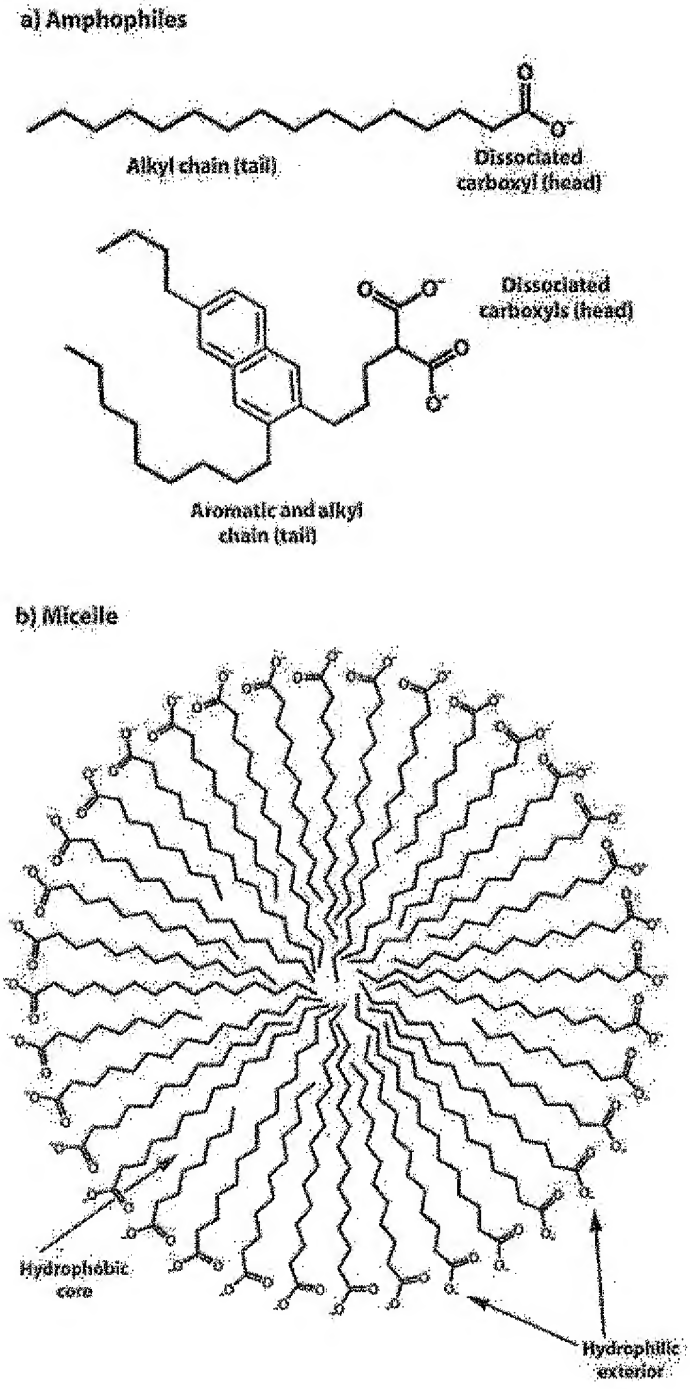


FIGURE 4.25 The self-aggregation theory of humic substance production is based on the premise that biopolymer degradation products are amphophilic, consisting of hydrophilic (polar or ionic) and hydrophobic molecular segments (a). In solution, the degradation byproducts form micelle-like structures (b), composed of a hydrophilic outer layer and a hydrophobic core. Accumulation of humic substances on soil surfaces (c) is initiated by the reaction of hydrophilic moieties with surface oxygens (electrostatic interactions) or surface metal cations (direct ionic/covalent bonds). The hydrophobic portions of these surface molecules are shielded from the polar aqueous phase by a second layer of amphophiles to form a bilayer.

c) Bilayer

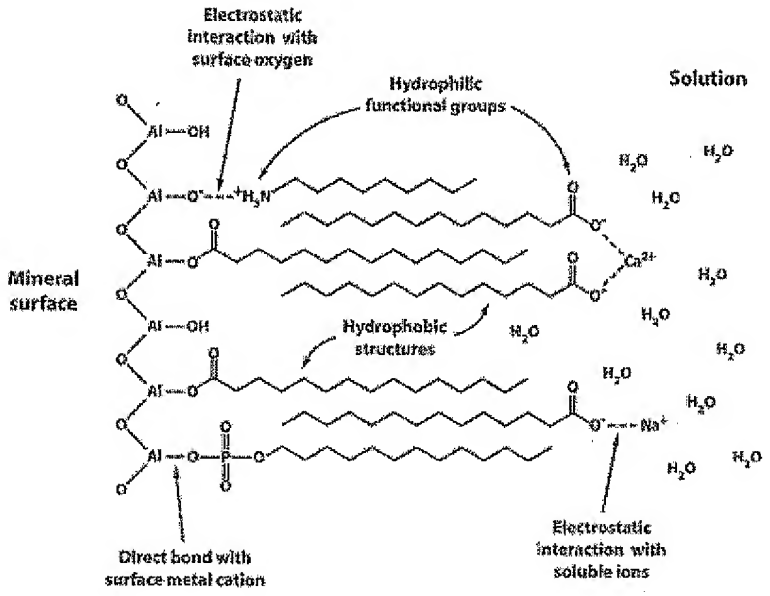


FIGURE 4.25 (*continued*).

Two mechanisms have been proposed to explain supramolecule formation on soil surfaces. One theory suggests that polar portions of the amphophilic molecules, the dissociated carboxyl group, reacts with inorganic, constant potential surface functional groups via a ligand exchange mechanism (described in Chapter 7) to form direct and relatively covalent bonds with surface metal cations (Figure 4.25c). Adsorption may also occur if the surface and the organic moiety have opposing charges, as illustrated by the electrostatic retention of a protonated amino group by a negatively charged surface site. The hydrophobic portions of these surface-bound molecules are then stabilized by the additional accumulation of amphophiles at the surface, arranged such that the polar portions point toward the aqueous phase. The resulting organic agglomeration at the soil surface is called a bilayer.

A second theory that describes the accumulation of solid humic substances assigns relatively unique hydrophilic-hydrophobic character to the different humus fractions. Fulvic acids are described as associations of small hydrophobic molecules that contain a sufficient number of acidic functional groups to maintain supramolecule solubility at any solution pH (again, the operational definition of fulvic acid). Humic acids are described as supramolecular associations composed of predominantly hydrophobic units that are stabilized by weak attractive forces. As solution pH decreases, intermolecular hydrogen bonding stabilizes the units further such that precipitation occurs (acid insoluble). These hydrophobic units may also bond to clay minerals that have a small amount of isomorphic substitution and/or an interlayer with hydrophobic character, such as the smectites (see Chapter 7).

Arguments for and against the view that humic substances are supramolecular entities are quite persuasive (for examples, see Swift (1999) and Burdon (2001)). However, it is important to recognize that humic substances are not generated by any one single pathway, but by several pathways with one generally dominating. For example, in poorly drained soils or anaerobic systems lignin decomposition is severely restricted. In these environments, the humic substances may arise principally via pathways 3 and 4 (Figure 4.22). However, in well-aerated soils almost all of the

lignin and other biopolymers may be completely degraded to their base monomers with only a small amount of larger biopolymer fragments repolymerizing with the monomers to form humic substances (pathway 2). Further, it is entirely conceivable that monomers and larger fragments may self-associate or form associations with secondary synthesis products through weak van der Waals and electrostatic forces (pathway 5), irrespective of the environmental conditions.

4.4.2 Chemical and Structural Characteristics of Humic Substances

As discussed in the previous section, humic substances are either exceedingly complex macromolecules formed by secondary abiotic or biotic synthesis reactions that repolymerize the polymeric fragments or monomeric units of biopolymers; or, they are associates, aggregates, or micelles of relatively small molecules held together by weak van der Waals forces and hydrogen bonding; or, they are all of the above. Irrespective of their mechanisms of formation or their structural character, humic substances are most often studied after they have been isolated and purified according (typically) to the extraction procedure detailed in Figure 4.21. Within this framework, a considerable amount of chemical information, such as elemental and functional group composition, has been accumulated. However, the macrostructural characteristics of humic substances remain an enigma.

4.4.2.1 Elemental Content

One of the most common means of characterizing humic substances is to determine the total elemental content. Typically, this involves the determination of C, H, O, N, and S (and sometimes P) concentrations in the isolates. The separates that comprise the humic substances tend to differ in their elemental character (Table 4.2). Relative to fulvic acids, humic acids generally have greater C and lower O content. The disparity between C and O concentrations also results in differences in the O/C mole ratios between the two fractions, with fulvic acids having a greater O/C ratio than the humic acids. The greater oxygen content and O/C ratio of the fulvic acids is related to the

TABLE 4.2
Mean Elemental Content (in g kg^{-1}), O/C and H/C Mole Ratios (in mol mol^{-1}) of Soil Humic and Fulvic Acids Collected from Around the World

	Humic Acids		Fulvic Acids	
Element	Mean	Range	Mean	Range
C	562	536–587	457	407–506
H	47	32–62	54	38–70
O	355	328–383	448	397–498
N	32	8–43	21	9–33
S	8	1–15	19	1–36
O/C[a]	0.51		0.74	
H/C[a]	1.00		1.42	

[a] The mole ratio values were computed using the mean elemental contents of C, O, and H.

From Steelink, C. Elemental characteristics of humic substances. *In* G.R. Aiken *et al.* (ed.) *Humic Substances in Soil, Sediment and Water*. John Wiley & Sons, New York, 1985, pp. 457–476. With permission.

higher concentrations of oxygen containing functional groups, such as the carboxyl group (R—COOH), and to the higher concentrations of carbohydrates in the fulvic acid materials. The higher concentrations of oxygen-bearing moieties in the fulvic acids is also expected and related to their operational definition. A relatively large number of acidic groups are required to maintain fulvic acid solubility when the initially alkaline soil extract is acidified (Figure 4.21). Another distinguishing characteristic of the fulvic and humic acids is the H/C mole ratio. The relative magnitude of the H/C mole ratio is used to indicate the degree of aromaticity and unsaturation of carbon chains. Relatively small H/C mole ratios suggest that the humic substances have a high degree of aromaticity; whereas, larger values indicate a greater abundance of aliphatic structures. Typically, H/C mole ratios for the fulvic acids are greater than those of the humic acids, indicating that the fulvic acids have greater aliphatic character than the humic acids (or that the humic acids have greater aromatic character than the fulvic acids).

Rice and MacCarthy (1991) surveyed the elemental contents of humins, humic acids, and fulvic acids extracted from soil, freshwater, marine, and peat sources from around the world (Table 4.3). One of the more interesting aspects of the data compiled by these researchers is the distribution of the concentration and mole ratio data about their respective mean values. As illustrated in Table 4.3, the observed ranges of elemental composition data for the three humic substances are quite broad. For example, the C content of one humic acid sample contained 372 g C kg^{-1}, while another contained 758 g C kg^{-1}. Similarly, the fulvic acids contained from 351 to 754 g C kg^{-1}, and the humins from 483 to 616 g C kg^{-1}. This can be explained given that these humic substances are products of numerous and variable extraction procedures (but based on the common theme shown in Figure 4.21) performed by many different individuals, and that the isolates are derived from a variety of sources (soils, freshwater, marine, and peat) from different geographical locations and parent materials (vegetation). It would appear from these data that the different humic substances (humic acids, fulvic acids, and humins) are chemically diverse in nearly all elemental respects, and on average chemically indistinguishable from one another, because their elemental concentration ranges overlap (as illustrated for carbon above). However, it must be recognized that these data values are the extremes and do not represent the respective populations of the humic substances; they are the outliers.

Figure 4.26 illustrates the normal distribution of the elemental contents for the humins, humic acids, and fulvic acids computed using the mean and standard deviation data reported by Rice and MacCarthy (1991). Also illustrated are the 95% confidence intervals of the mean values. The x-axis represents the elemental concentration (or mole ratio), and the y-axis represents the number of samples (or frequency of occurrence) having elemental contents that are a particular concentration (the curves model the histograms of the actual data). Instead of a broad and diffuse distribution that might be expected given that the data are obtained from diverse sources by diverse means, it is observed that the elemental contents of the substances are normally distributed about mean values that have relatively small standard deviations (with the exception of the S data). For example, the mean C content of the humic substances is 551 g kg^{-1} with a standard deviation of 50 g kg^{-1}. Thus, 68% of the 410 humic acid samples examined have C concentrations in the 501 to 601 g kg^{-1} range (±1 standard deviation); 96% of the 410 samples have C concentrations within the 451 to 651 g kg^{-1} range (±2 standard deviation).

Humic substances are compositionally unique relative to the environmental conditions in which they form. However, based on the statistical evaluation of Rice and MacCarthy (1991), the mean chemical compositions (concentrations of C, H, O, N, and S) and the mole ratios (O/C and H/C) of humic acids and humins are statistically similar (the 95% confidence intervals for the mean values overlap, as shown in Figure 4.26). The fulvic acids differed from the humic acids with respect to their mean C, N, and O concentrations and O/C and H/C ratios. On average, the fulvic acids contain less C than humic acids (462 g kg^{-1} vs. 551 g kg^{-1}), more O (456 g kg^{-1} vs. 356 g kg^{-1}), and less N (25 g kg^{-1} vs. 35 g kg^{-1}) (Table 4.3). The O/C mole ratio of the fulvic acids is also greater than that of the humic acids (0.76 mol mol^{-1} vs. 0.50 mol mol^{-1}). The mean concentrations of C, O, and

TABLE 4.3
Mean Elemental Content (in g kg^{-1}) and O/C and H/C Mole Ratio Values (in mol mol^{-1}) of Humic Acids, Fulvic Acids, and Humin Obtained from a Geographically Diverse Range of Soil, Freshwater, Marine, and Peat Sources[a]

	C	H	O	N	S	O/C	H/C
				Humic Acids			
All sources (n = 410)[b]	551 ± 50 (372–758)	50 ± 11 (16.4–117)	356 ± 58 (79.3–566)	35 ± 15 (5.0–105)	18 ± 16 (1–83)	0.50 ± 0.13 (0.08–1.20)	1.10 ± 0.25 (0.08–1.85)
Soil (n = 215)	554 ± 38 (372–641)	48 ± 10 (16.4–80)	360 ± 37 (271–520)	36 ± 13 (5.0–70.0)	8 ± 6 (1–48.8)	0.50 ± 0.09 (0.33–0.97)	1.04 ± 0.25 (0.08–1.77)
Freshwater (n = 56)	512 ± 30 (438–560)	47 ± 6 (35–65.4)	404 ± 38 (309–482)	26 ± 16 (6.3–79.7)	19 ± 14 (3.5–43.1)	0.60 ± 0.08 (0.42–0.80)	1.12 ± 0.17 (0.79–1.69)
Marine (n = 95)	563 ± 66 (375–758)	58 ± 14 (37.6–117)	317 ± 78 (79.3–566)	38 ± 15 (9.7–105)	31 ± 14 (12–83)	0.45 ± 0.18 (0.08–1.20)	1.23 ± 0.23 (0.67–1.85)
Peat (n = 23)	571 ± 25 (505–628)	50 ± 8 (36–65.7)	352 ± 27 (307–432)	28 ± 10 (6.0–39)	4 ± 2 (1–7)	0.47 ± 0.06 (0.37–0.64)	1.04 ± 0.17 (0.73–1.35)
				Fulvic Acids			
All sources (n = 214)	462 ± 54 (351–754)	49 ± 10 (4.3–72)	456 ± 55 (169–558)	25 ± 16 (4.5–81.6)	12 ± 12 (1.0–36)	0.76 ± 0.16 (0.17–1.19)	1.28 ± 0.31 (0.77–2.13)
Soil (n = 127)	453 ± 54 (351–754)	50 ± 10 (32–70.0)	462 ± 52 (169–559)	26 ± 13 (4.5–58.7)	13 ± 11 (1–36)	0.78 ± 0.16 (0.17–1.19)	1.35 ± 0.34 (0.77–2.13)
Freshwater (n = 63)	467 ± 43 (392–563)	42 ± 7 (4.3–59)	459 ± 51 (347–558)	23 ± 21 (4.7–81.6)	12 ± 9 (1.6–30.5)	0.75 ± 0.14 (0.49–1.07)	1.10 ± 0.13 (0.81–1.53)
Marine (n = 12)	450 ± 40 (384–500)	59 ± 9 (43–68.0)	451 ± 60 (369–545)	41 ± 23 (10–68.3)	—	0.77 ± 0.17 (0.55–1.07)	1.56 ± 0.13 (1.31–1.73)
Peat (n = 12)	542 ± 43 (469–608)	53 ± 11 (42–72)	378 ± 37 (311–443)	20 ± 5 (12–26)	8 ± 6 (12–26)	0.53 ± 0.094 (0.38–0.71)	1.20 ± 0.33 (0.85–1.84)
				Humins			
All sources (n = 26)	561 ± 26 (483–616)	55 ± 10 (42–72.8)	347 ± 34 (288–451)	37 ± 13A (9.0 – 60.0)	4 ± 3 (1–9)	0.46 ± 0.06 (0.37–0.61)	1.17 ± 0.24 (0.82–1.72)

[a] Mean ± standard deviation (range is shown in parentheses).

[b] Within each separate, the data are either aggregated (as in the "All sources" row) or separated by sample type (soil, freshwater, marine, or peat). The n values represent the number of samples included in the mean, standard deviation, and range. However, the n values differ for S in all categories and for N in some categories. For the humic acids, the n values for S are: all sources, n = 160; soil, n = 67; freshwater, n = 13; marine, n = 66; peat, n = 12. For N in peat humic acids, n = 21. For the fulvic acids, the n values for S are: all sources, n = 71; soil, n = 45; freshwater, n = 14; marine, n = 12; peat, n = 11. For the humins, n = 24 for N data and n = 16 for S data.

From Rice, J.A. and P. MacCarthy. Statistical evaluation of the elemental composition of humic substances. *Org. Geochem.* 17:635–648, 1991. With permission.

N, and the O/C ratio of fulvic acids also differ from those of the humins, with the fulvic acids containing less C, more O, less N, and having a higher O/C. Finally, soil fulvic acids have lower C concentrations than soil humic acids (453 g C kg^{-1} vs. 554 g C kg^{-1}), lower N concentrations (26 g N kg^{-1} vs. 36 g N kg^{-1}), higher O concentrations (462 g O kg^{-1} vs. 360 g O kg^{-1}), higher O/C mole ratios (0.78 vs. 0.50), and higher H/C mole ratios (1.35 vs. 1.04) (Table 4.3).

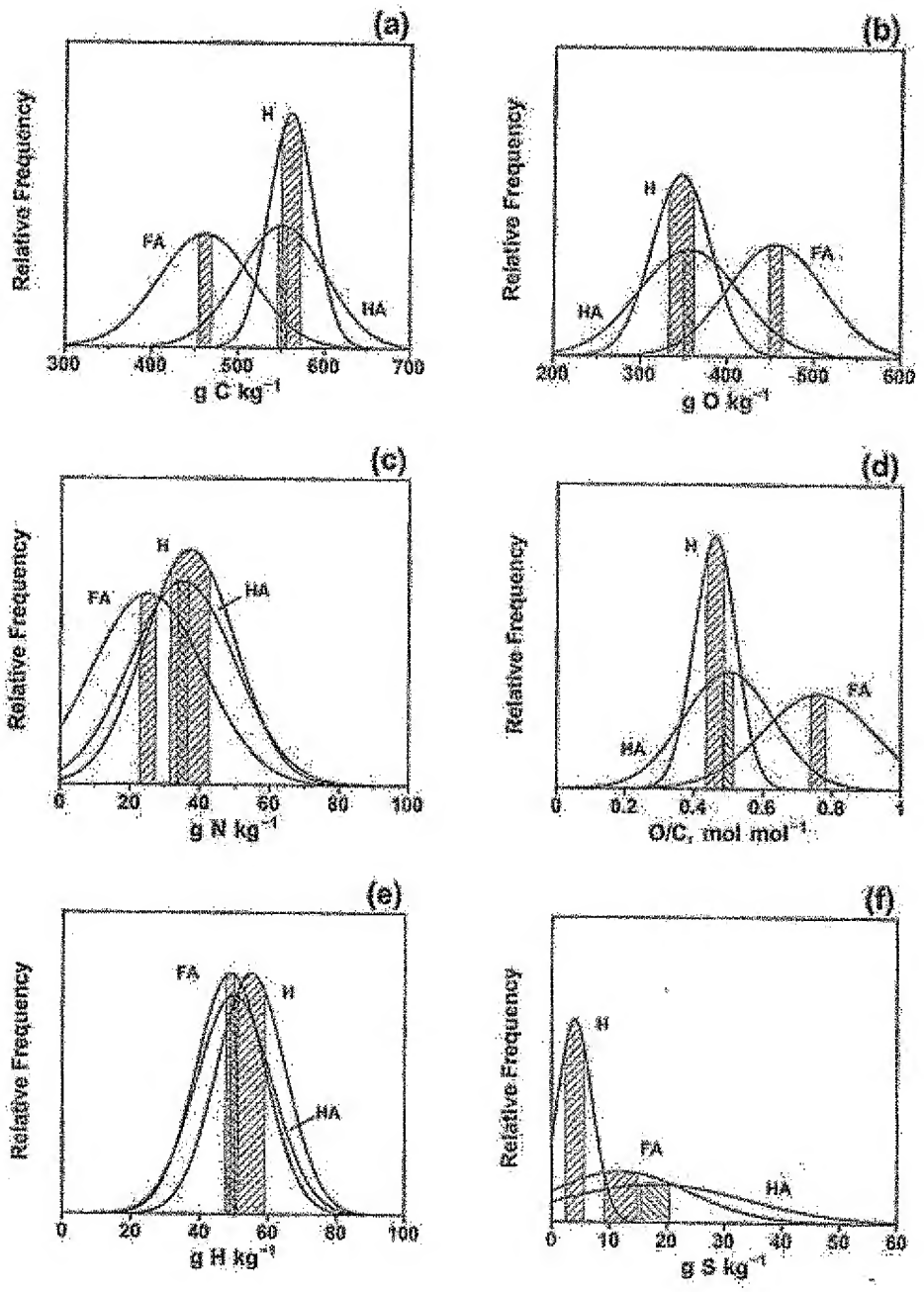


FIGURE 4.26 Frequency diagrams illustrating the normal distribution of elemental composition data for the humic substances. The solid lines represent the normal distribution curves produced using the statistical data of Rice and MacCarthy (1991). The hatched areas represent the 95% confidence intervals about the mean values. The concentrations are presented on a g kg^{-1} of humic substance basis: (a) organic carbon; (b) oxygen; (c) nitrogen; (d) mole ratio of oxygen to carbon; (e) hydrogen; (f) sulfur; and (g) mole ratio of hydrogen to carbon.

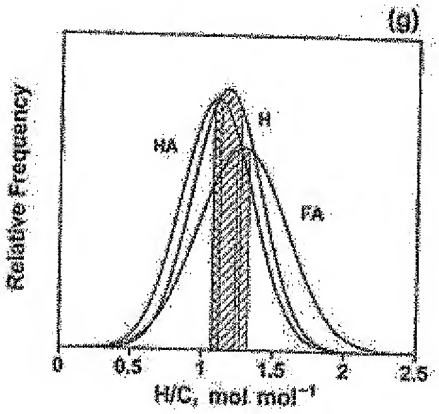


FIGURE 4.26 (*continued*).

The segregation of the humic and fulvic acids by source tends to reduce the variability associated with the mean composition data (standard deviations are decreased). Further, compositional differences within the humic and fulvic acids as a function of source may also be illustrated. Humic acids obtained from different environments differ in their elemental contents. On average, soil humic acids have higher C (554 g kg^{-1} vs. 512 g kg^{-1}) and N (36 g kg^{-1} vs. 26 g kg^{-1}) contents than freshwater humic acids, and lower O (360 g kg^{-1} vs. 404 g kg^{-1}) contents and O/C (0.50 vs. 0.60) ratios (Table 4.3). Degree of aliphaticity of humic acids also decreases from freshwater (H/C = 1.12) to soil sources (H/C = 1.04). Within the fulvic acids, soil sources are more aliphatic than freshwater sources (H/C is 1.35 for soil and 1.10 for freshwater).

4.4.2.2 Functional Groups and Structural Components

The major functional groups in humic substances are oxygen containing and include carboxyls, alcoholic and phenolic hydroxyls, carbonyls, and methoxyls. These groups are illustrated in Figure 4.3. Techniques for determining the types and concentrations of the various functional groups in humic substances can be grouped into two categories: wet-chemical methods and spectroscopic procedures. Typically, wet-chemical methods exploit the acidic properties of the various functional groups, or their derivatives. The more commonly used wet-chemical methods are described in Table 4.4. Many of the methods suffer from an inability to react specifically with the target functional groups on the humic macromolecular structure. For example, a common method for determining the carboxyl content of humic substances is the calcium acetate procedure. In this method, a 10-mL volume of a standard 0.5 *M* calcium acetate solution is reacted with a 50- to 100-mg mass of humic substance. After a 24-h reaction period, the humic residues are quantitatively separated from the solution by filtration and the solution titrated with standard 0.1 *M* NaOH. The excess volume of NaOH required to attain a pH of 9.8 in the humic filtrate, relative to the volume needed to attain pH 9.8 in a 0.5 *M* calcium acetate blank, is directly related to the concentration of acid functional groups. Although this is a very straightforward analytical procedure, there are a number of confounding factors that may lead to questionable analytical results. A major interference may arise from the dissociation of acidic hydroxyl groups. Normally, hydroxyl groups are only weakly acidic. For example, phenol and most substituted phenols have pK_a values that range between 9 and 10. However, hydroxyl groups in humic substances are attached to a diverse variety of aliphatic and aromatic structures. These different structures influence the Lowry-Brønsted acidity of the hydroxyls such that the groups may display pK_a values that are less than the pH of the calcium acetate solution (approximately 9).

TABLE 4.4
Summary of the Wet-Chemical Methods Commonly Employed to Characterize Various Functional Groups in Humic Substances

Functional Group	Method	Reaction	Analysis	Main Interferences
Total acidity	Barium hydroxide	$2(R{-}COOH + R{-}OH + Ar{-}OH) + Ba(OH)_2 \rightarrow (R{-}COO + R{-}O + Ar{-}O)_2Ba(s) + 2H_2O$	Titration of unused $Ba(OH)_2$ with standard acid	1) Not all humic material may be precipitated 2) Excess mineral acids from humic substance isolation may be present
Carboxyl	Calcium acetate	$2R{-}COOH + Ca(CH_3COO)_2 \rightarrow (R{-}COO)_2Ca + 2CH_3COOH$	Titration of liberated acetic acid with standard base	Reaction with acidic OH groups occurs
	Direct titration	$R{-}COOH + NaOH \rightarrow R{-}COONa + H_2O$	NaOH consumed to reach pH 8 is the carboxyl content	Assumes that only carboxyl groups have pK_a values less than 7
Total-OH	Acetylation	$(R{-}OH + Ar{-}OH) + (CH_3CO)_2O \rightarrow (R{-}O{-}C(=O){-}CH_3 + Ar{-}O{-}C(=O){-}CH_3) + CH_3COOH$	Titration of liberated acetic acid with standard base	Significant acetylation of carboxyl groups occurs requiring a correction
Phenolic-OH	Difference	[Phenolic-OH] = [total acidity] − [R—COOH]		Compounded errors
	Ubaldini method	1) $Ar{-}OH + KOH \rightarrow Ar{-}O{-}K + H_2O$ 2) $2Ar{-}O{-}K + CO_2 + H_2O \rightarrow 2Ar{-}OH + K_2CO_3$	Titration of K_2CO_3 with standard acid	1) Proton-K exchange reaction is nonspecific 2) Hydrolysis by KOH Compounded errors
Alcoholic-OH	Difference	[Alcoholic-OH] = [total-OH] − [phenolic-OH]		
Total carbonyl	Derivitization to oxime using hydroxylamine	$RC{=}O + NH_2OH \rightarrow RC{=}NOH + H_2O$	Titration of unused hydroxylamine using standard perchloric acid	Reagent may react with groups other than $RC{=}O$
Quinones	Ferrous iron reduction in alkaline triethanolamine	$Ar{=}O + Fe^{2+} + H^+ \rightarrow Ar{-}OH + Fe^{3+}$	Titration of unused ferrous Fe with standard chromate	
Ether linkages	Zeisel method	1) $(R{-}OCH_3 + Ar{-}OCH_3) + HI \rightarrow (R{-}OH + Ar{-}OH) + CH_3I$ 2) $CH_3I + 6Br_2 + 5H_2O \rightarrow HIO_3 + 12HBr + CO_2$ 3) $2HIO_3 + 10KI + 5H_2SO_4 \rightarrow 6I_2 + 6H_2O + 5K_2SO_4$	Titration of liberated I_2 with standard sodium thiosulfate ($Na_2S_2O_3$) with starch as an indicator	

TABLE 4.5
Mean Total Acidity and Functional Group Content (in mol kg^{-1}) of Soil Humic and Fulvic Acids Collected from around the World

Functional Group	Humic Acids		Fulvic Acids	
	Mean	Range	Mean	Range
Total acidity	67	56–89	103	64–142
Carboxyl	36	15–57	82	52–112
Phenolic-OH	39	21–57	30	3–57
Alcoholic-OH	26	2–49	61	26–95
Carbonyl	29	1–56	27	13–42
Methoxyl	6	3–8	8	3–12

From Stevenson, F.J. *Humus Chemistry: Genesis, Composition, Reactions*. John Wiley & Sons, New York, 1994. With permission.

Thus, these hydroxyls are ionized (as are the carboxyl groups) and they dissociate in the alkaline calcium acetate solution. An additional confounding factor is that the Ca^{2+} is capable of displacing protons from a small number of protonated functional groups that would otherwise not deprotonate in the alkaline solution if, for example, Na^{+} were the principal cation. Both interferences tend to overestimate the carboxyl content of humic substances.

Despite the questionable selectivity of the various wet-chemical methods, they do provide information on the nature and trends of the distribution of functional groups in the various humic substances. The humic and fulvic acids differ relative to their compositions of oxygen functional groups (Table 4.5). On average, humic substances contain approximately equal contents of carboxyl (36.0 mol kg^{-1}) and phenolic-OH (39.0 mol kg^{-1}) groups. Although the carboxyl and phenolic-OH groups appear to dominate, the abundances of alcoholic-OH and carbonyl groups are also highly significant (26.0 and 29.0 mol kg^{-1}). Fulvic acids are dominated by carboxyl groups (82.0 mol kg^{-1}), followed by alcoholic-OH (61.0 mol kg^{-1}). As a result of the greater carboxyl content, the fulvic acids have greater acidity (103.0 mol kg^{-1}) than the humic acids (67.0 mol kg^{-1}). The concentrations of the phenolic-OH and carbonyl groups are approximately equal in the fulvic acids (30.0 and 27.0 mol kg^{-1}), but are substantially lower than the carboxyl and alcoholic-OH groups. Methoxyl groups (OCH_3) are minor components of both the fulvic and humic acids.

Studies of the structural components of the humic substances, as well as their functional group contents, are most commonly performed using nondestructive spectroscopic techniques. Chemical degradation (destructive) methods, which employ pyrolysis (heat), acids, oxidants, or reductants (or some combination) to cleave humic structures into smaller units for analysis, have also been extensively employed to provide information on the structural character of humic substances. Once produced, the relatively simple compounds created by the degradation process are related back to the original macromolecular structure (either directly or through deduction based on potential degradation mechanisms). Although some feel that the degradation methods provide the best descriptions of humic structures, their popularity has been supplanted by nondestructive techniques.

Of the spectroscopic techniques, which include the ultraviolet-visible, fluorescence, and infrared spectroscopy, nuclear magnetic resonance (NMR) spectroscopy provides the most definitive information on the structural components and functional groups in humic substances. Further, NMR provides semiquantitative and quantitative information and is applicable to the analysis of both liquid- and solid-state samples. Nuclear magnetic resonance exploits the property of nuclear spin. A magnetic moment is associated with nuclear spin, such that the nuclei behave like tiny magnets. In the absence of a magnetic field, the poles of these nuclear magnets are randomly aligned.

TABLE 4.6
Chemical Shift Ranges and Assignments Associated with Cross-Polarization, Magic Angle Spinning ^{13}C Nuclear Magnetic Resonance (CPMAS ^{13}C NMR) Using a Tetramethylsilane Standard (the Regions Are Illustrated in Figure 4.27)

Chemical Shift, ppm	Assignments
0–25	Primary alkyl ($-CH_3$)
25–35	Secondary alkyl ($-CH_2-$)
35–50	Complex aliphatic (CH_2CH, C)
50–60	Methoxyl, methyne, tertiary and quaternary alkyls ($-OCH_3$, CH–NH, CH, C)
60–96	Saccharide, alcohol, ether (CHOH, CH_2OH, CH_2-O-)
96–108	Anomeric (O–C–O)
108–120	Aromatic (=CH=)
120–145	Aromatic (=CH=, =C–)
145–162	Phenolic (C–O–, C–OH)
162–190	Carboxyl, ester, quinone (COOH, COO–, C=O)
190–220	Ketone, aldehyde, quinone (C=O, HC=O)

However, when a magnetic field is imposed, certain atomic nuclei align with the field in only one of two ways: with the field (low energy, +½ spin) or against the field (high energy, −½ spin). The nuclei that have spin ±½, and that are most important to the characterization of humic substances, are ^1H, ^{13}C, ^{15}N, and ^{31}P. Nuclei that are placed in a magnetic field will predominantly exist in the low energy, +½ spin state. However, transition of the nuclei to the high energy spin state (the resonance condition) can be achieved by imposing an oscillating magnetic field of electromagnetic radiation that is perpendicular to a steady magnetic field, and that has a frequency that corresponds to the energy separation of the +½ and −½ spin states. The frequency required to achieve the resonance is a function of the chemical environment in which a nucleus resides, as the atoms that surround a nucleus provide shielding from the magnetic field. Thus, there is a shift in the resonance frequency relative to a standard. For ^{13}C NMR the standard compound is commonly tetramethylsilane (TMS, $(CH_3)_4Si$). The degree of shift is indicative of the types and arrangement of the surrounding atoms. The chemical shift between the resonance frequency of the standard and that of a moiety in the compound of interest is denoted by the symbol δ and is expressed in units of parts per million (ppm).

Perhaps the most common NMR technique employed to characterize humic substances is a solid-state ^{13}C NMR method called cross-polarization, magic angle spinning ^{13}C NMR (CPMAS ^{13}C NMR). The chemical shift assignments, relative to TMS, for CPMAS ^{13}C NMR are given in Table 4.6 and the example spectra in Figure 4.27. Quantitative analysis is performed by determining the area under the spectra that is defined by a given chemical shift region. This area is then divided by the total peak area of the spectra to yield the fractional amount of C associated with the particular chemical shift region. Mahieu et al. (1999) surveyed the published CPMAS ^{13}C NMR data for soil humic substances obtained from different environments (Table 4.7). On average, the alkyl and O-alkyl content of humic and fulvic acids are similar. However, the humic acids have a greater abundance of aromatic C than the fulvic acids (25.4 mol kg^{-1} vs. 19.1 mol kg^{-1} of organic C), while the fulvic acids have a greater abundance of carbonyl C (20.6 mol kg^{-1} vs. 13.7 mol kg^{-1} of organic C). These findings are generally consistent with the wet-chemical data presented in Table 4.5. However, the greater abundance of alcoholic-OH functional groups in the fulvic acids, determined by wet-chemical methods, is not confirmed by the CPMAS ^{13}C NMR data; that is, a greater abundance of O-alkyl C in fulvic acids relative to humic acids is not seen by CPMAS ^{13}C NMR. Ussiri and Johnson (2003) performed a direct comparison of CPMAS ^{13}C NMR spectra for humin,

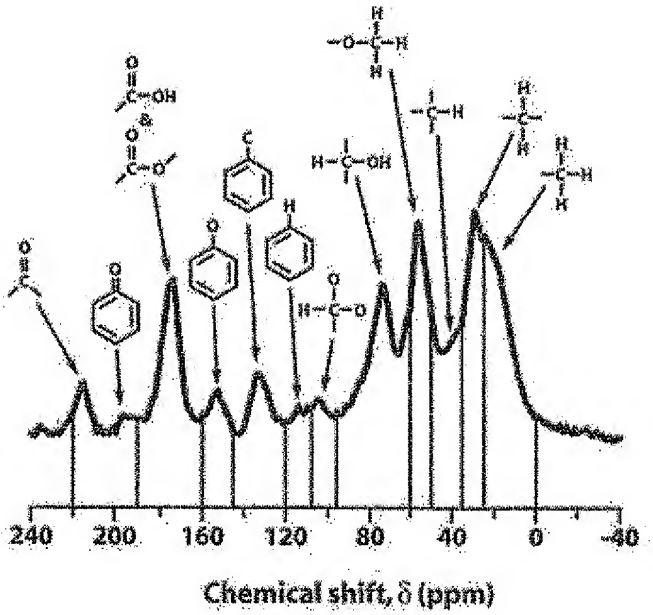


FIGURE 4.27 Example of a cross-polarization, magic angle spinning ^{13}C nuclear magnetic resonance (CPMAS ^{13}C NMR) spectra of humic acid from Conte et al. (1997). The vertical lines delineate the typical chemical shift assignments (from Table 4.6).

TABLE 4.7
Structural Composition (Mean ± Standard Deviation in mol kg^{-1} of Organic Carbon) of Soil Humic and Fulvic Acids in Soils Collected from a Wide Variety of Climates Using Cross-Polarization, Magic Angle Spinning (CPMAS) ^{13}C NMR

Functional Groups[a]	Humic Acids (n = 208)[b]		Fulvic Acids (n = 66)	
	Mean	Range	Mean	Range
Alkyl	22.1 ± 9.16	8.33–58.4	22.1 ± 8.33	6.99–39.6
O-Alkyl	22.1 ± 6.58	0–40.8	21.6 ± 9.91	7.24–50.8
Aromatic	25.4 ± 7.41	7.41–57.4	19.1 ± 5.38	5.83–30.7
Carbonyl	13.7 ± 3.75	3.33–25.0	20.6 ± 3.66	1.25–28.1

[a] Alkyls, between 0 and 50 ppm; O-alkyls, between 50 and 110 ppm; aromatics, between 110 and 160 ppm; carbonyls, between 160 and 220 ppm. See Table 4.6 for the specific carbon types included in each chemical shift region.

[b] *n* denotes the number of samples included in the statistical analysis.

From Mathieu, N., D.S. Powlson, and E.W. Randall. Statistical analysis of published carbon-13 NMR spectra of soil organic matter. *Soil Sci. Soc. Am. J.* 63:307–319, 1999. With permission.

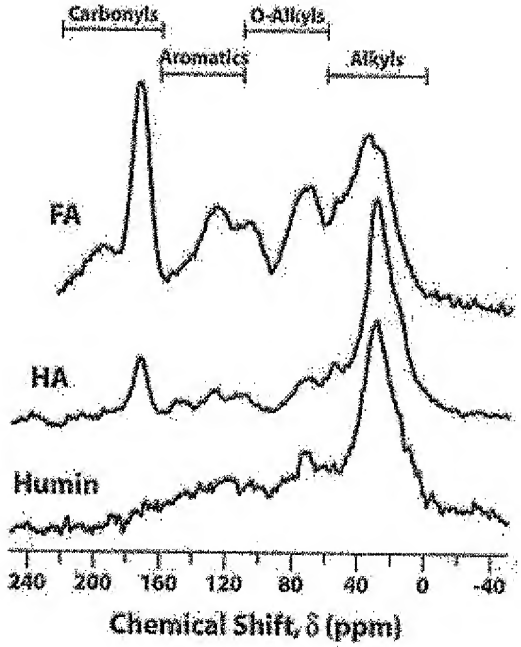


FIGURE 4.28 A comparison of the chemical characteristics of carbon in humic substance isolates from the Bh horizon of a Typic Haplorthod (65.3 g kg^{-1} SOC) determined by cross-polarization, magic angle spinning ^{13}C nuclear magnetic resonance (CPMAS ^{13}C NMR) (modified from Ussiri and Johnson, 2003). The four general chemical shift regions are alkyl C (0–50 ppm), O-alkyl C (50–110 ppm), aromatic C (110–160 ppm), and carbonyl C (160–220 ppm). See Table 4.6 for specific carbon types included in each shift region.

humic acid, and fulvic acid obtained from the Bh horizon of a Typic Haplorthod. The varied chemical nature of the three separates is illustrated in Figure 4.28, and in the discussion that follows. Carbon in the humin fraction (residuum remaining after base extraction of SOM) of the Bh horizon is principally found in alkyl structures, such as n-alkanes, fatty acids, and waxes (440 g kg^{-1} of C), followed by O-alkyls (297 g kg^{-1} of C), aromatics (213 g kg^{-1} of C), and carbonyls (57 g kg^{-1} of C). In the humic acid fraction, alkyl structures contain 533 g kg^{-1} of C, followed by O-alkyls (207 g kg^{-1} of C), aromatics (130 g kg^{-1} of C), and carbonyls (133 g kg^{-1} of C). Both the humin and humic acids substances are dominated by alkyl carbon; however, the humic acid fraction contains less carbon in O-alkyl and aromatic structures, and more C in carbonyls. Finally, the fulvic acid fraction of the Bh horizon contains the most C in alkyl structures (343 g kg^{-1} of C), followed by O-alkyls (279 g kg^{-1} of C), carbonyls (228 g kg^{-1} of C), and aromatics (161 g kg^{-1} of C). Again, the fulvic acid fraction contains a greater abundance of oxygen-bearing functional groups (507 g kg^{-1} of C; sum of O-alkyl and carbonyl concentrations) than either the humic acid (340 g kg^{-1} of C) or the humin fractions (354 g kg^{-1} of C).

Nuclear magnetic resonance has also been employed to characterize the forms of N and P in humic substances. Solid-state ^{15}N NMR spectra of humic substances are consistently similar, showing a major peak for amide N (–230 to 285 ppm relative to the nitromethane standard) which indicates the presence of proteinaceous materials (Figure 4.29). Other forms of organic N-containing structures are detected in humic substances, including indoles, pyrroles, and imidazoles (–140 to –250 ppm),

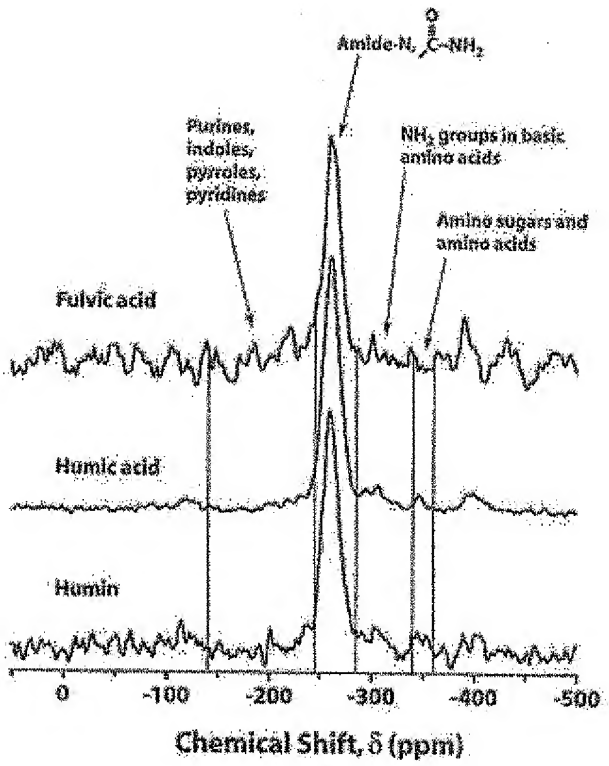


FIGURE 4.29 Example of a cross-polarization, magic angle spinning ^{15}N solid-state nuclear magnetic resonance (CPMAS ^{15}N NMR) spectra of the humic fractions extracted from plant residues (modified from Knicker, 2002). The general chemical shift regions and chemical assignments are also illustrated.

and amino groups (–285 to –320 ppm); however, these groups only occur in minor abundance. Liquid-state ^{31}P NMR (Figure 4.30) indicates the presence of both inorganic and organic P forms in humic substances. The most commonly identified forms of P are inorganic orthophosphate (6.0 to 7.5 ppm, referenced to an external 85% phosphoric acid solution), monoester organic P (found primarily in inositol phosphates) (4.2 to 6.0 ppm), diester organic P (–0.1 to –1.1 ppm), and organic pyrophosphates (–2.5 to –4.3 ppm). Other organic P forms that are detectable using ^{31}P NMR are the organic polyphosphates (–19 to –20 ppm) and the phosphonates (20 to 21 ppm). The liquid-state ^{31}P NMR spectra of a soil NaOH extract in Figure 4.29 (insert) indicates that the monoester P forms account for 79.4% of the total organic P (794 g kg^{-1} of P), while diester P and pyrophosphate P account for 4.5 and 5.1% of the total organic P. In general, liquid-state ^{31}P NMR studies indicate that monoester organic P accounts for approximately 75 to 85% of the total organic P extracted from soil with alkaline extractants.

4.4.2.3 Molecular Mass and Configuration

Humic substances are formed through the random polymerization or aggregation of a diverse array of compounds (monomers and fragments) from a pool comprised of the microbial degradates of biopolymers. The probability of finding two humic molecules that are exactly alike is exceedingly

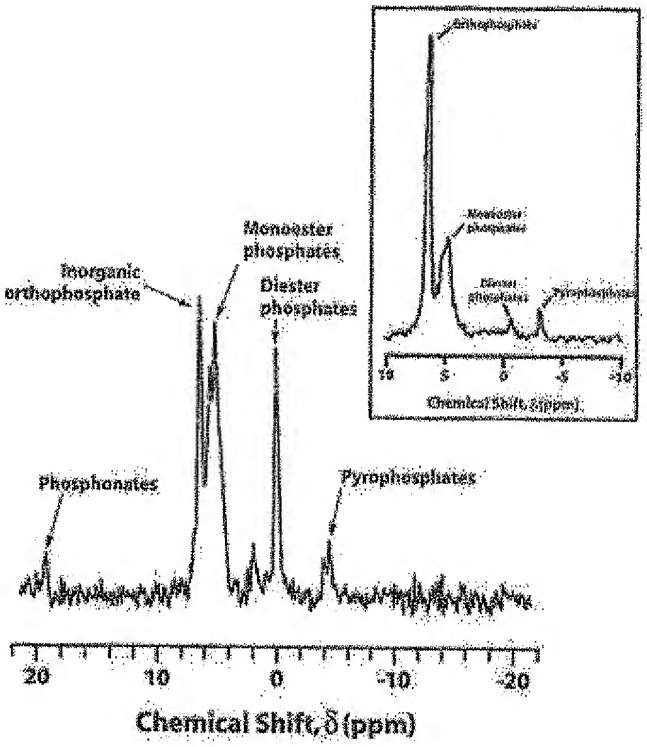


FIGURE 4.30 Liquid-state ^{31}P nuclear magnetic resonance spectra of NaOH extracts of an Alfisol and an Inceptisol from Hawkes et al. (1984) and Zhang et al. (1999) (insert).

small, particularly as molecules increase in size. In addition and despite their refractive nature, humic substances evolve and degrade, further enhancing their random character. Despite the randomness associated with their formation and degradation, there is relative uniformity among the average chemical properties of the humins and the humic and fulvic acids, such as elemental and functional group content (discussed in the previous sections). Indeed, these findings have been used to suggest that there exists an optimal chemical composition for humic substances in nature. However, the molecular masses of the humic substances do not appear to be constrained to the relatively narrow distributions exhibited by the chemical characteristics.

A number of methods have been employed to determine the molecular masses of humic substances. Ultrafiltration, small-angle x-ray scattering, and gel chromatography represent just a few of the techniques that have been employed (see Senesi and Loffredo [1999] for a review of these and other techniques). In general, soil humic acid extracts are composed of compounds that display a continuum of molecular masses ranging from approximately 1000 Da (daltons, a non-SI mass unit equal to one twelfth the atomic mass of ^{12}C; a dalton is equivalent to g mol^{-1}) to 500,000 Da, with the majority of substances having masses that center around 50,000 Da. Cameron et al. (1972) performed a pivotal study, the results of which have influenced the concepts of humic substance size and shape. They employed gel chromatography and pressure filtration through graded porosity membranes to fractionate the humic acid isolate from a Sapric Histosol. The molecular masses of the humic substances in each fraction were then determined by ultracentrifugation,

The molecular mass values for the humic acids ranged from approximately 2000 to 1,300,000 Da. Approximately 75% of the humic acids had molecular mass values <100,000 Da, and 25% had values <10,000 Da. A majority of the humic acids had molecular masses in the 20,000- to 50,000-Da range. Less than 20% of the substances were >100,000 Da in mass. Using their ultracentrifugation data, Cameron et al. (1972) convincingly proposed that humic acid macromolecules structurally consist of flexible, expanding, and branched random coils. Fulvic substances are quite small in comparison to the humic substances. Their masses tend to fall in a very limited range; from approximately 300 to 2000 Da.

Perhaps the most nebulous of characteristics of the humic substances is their molecular structure. It has been stated that any representation of the molecular configuration of a humic substance is essentially a cartoon. Humic scientists recognize that it is not possible to write a molecule structure or set of structures that truly defines the configuration of a humic substance. Structural representations are meant to convey information about the structural moieties that are thought to exist in humic substances, rather than represent the precise molecular structure. Such structures are called psuedostructures (after MacCarthy [2001]). A humic substance psuedostructure is a hypothetical molecular construct having elemental, structural, and functional group features that are consistent with some or all of the observed composition and mass properties. A pseudostructure of humic acid, developed by Schulten and Schnitzer (1997), is shown in Figure 4.31. Suwannee River fulvic acid psuedostructures have been proposed by Leenheer et al. (1998) (Figure 4.32a and b) and Kubicki and Apitz (1999) (Figure 4.32c).

The pseudostructure illustrated in Figure 4.31 was developed using the available chemical and structural information from the literature on humic acids. The pseudostructure contains numerous

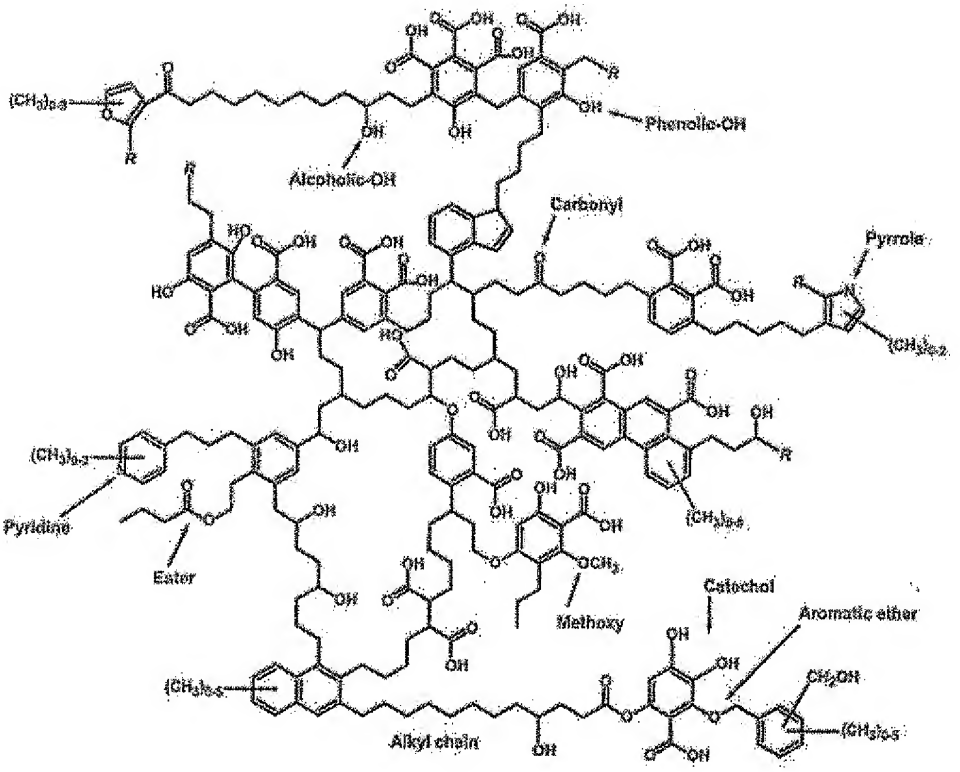


FIGURE 4.31 Partial pseudostructure of humic acid. (Modified from Schulten, H.R. and M. Schnitzer. Chemical model structures for soil organic matter and soils. *Soil Sci.* 162:115–130, 1997. With permission.)

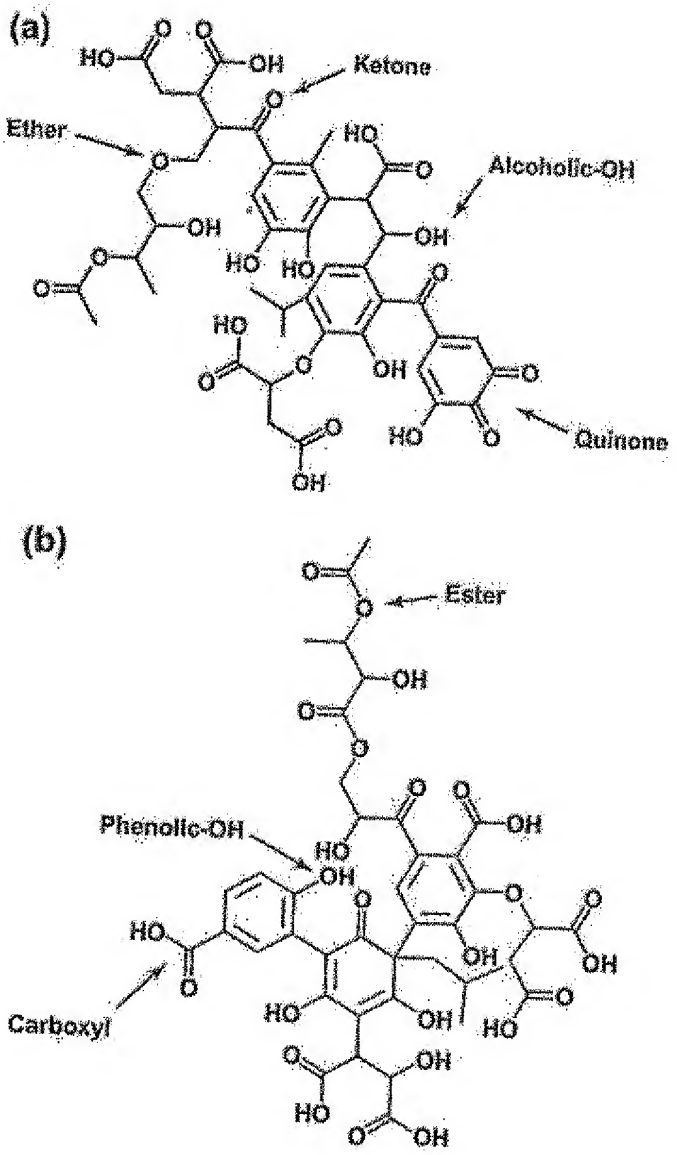


FIGURE 4.32 Pseudostructural models of Suwannee River fulvic acid. Fulvic acids in (a) and (b) are from Leenheer et al. (1998) and are based on different plant precursors. The model in (a) is derived from the degradation of proanthocyanidine and phloroglucinal tannins (Figure 4.17). The model in (b) is based upon a cutin-lignin-tannin complex. The pseudostructure in (c) is a three-dimensional representation of fulvic acid (Kubicki and Apitz, 1999).

oxygen-bearing structures and functional groups (carboxyls, phenolic and alcoholic hydroxyls, ketones, esters, and ethers), as well as a small number of N-bearing structures (pyrrole and pyridine). These groups are hydrophilic and are readily solvated (encircled by water molecules). Many of these groups, particularly the carboxyls and the N moieties, ionize and interact with inorganic and other organic ions or molecules from the aqueous phase. This type of interaction is responsible for the cation exchange capacity of SOM, which can range from 60 to 300 cmol kg^{-1} at pH 7, and account for 25 to 90% of the cation exchange capacity of mineral soils.

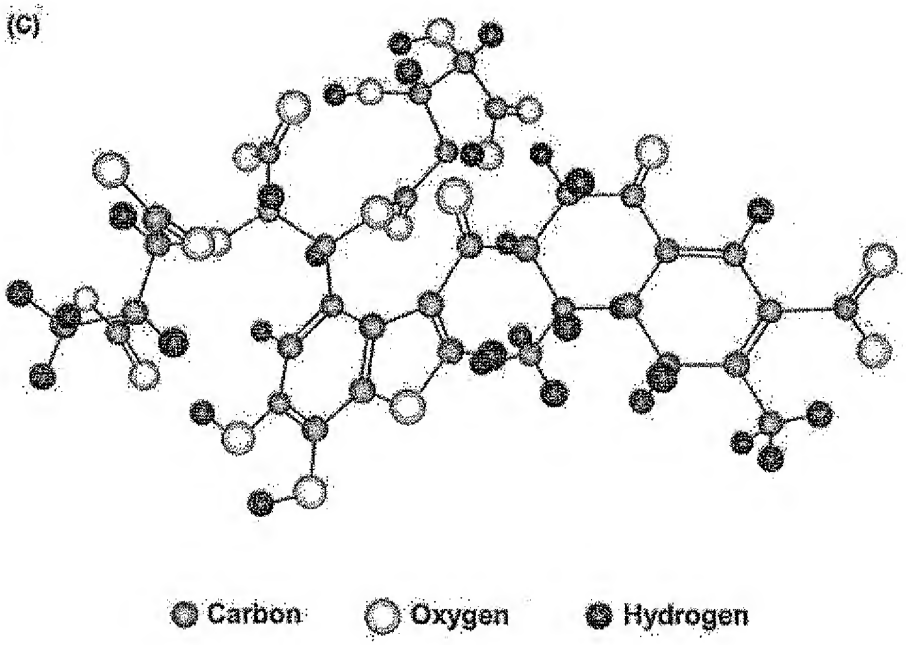


FIGURE 4.32 *(continued).*

The Suwannee River fulvic acid pseudostructures illustrated in Figure 4.32 were derived using measured chemical properties of the fulvic acid isolate. The fulvic acid pseudostructures are noticeably smaller than the humic acid structure (Figure 4.31). Indeed, the molecular mass of these fulvic acids is approximately 950 Da, while that of the humic acid described above is approximately 6650 Da. The fulvic acid pseudostructures characteristically have a large number of carboxyl groups, as well as alcoholic and phenolic hydroxyls, ester and ether linkages, and ketone groups. Relative to the humic acid structure, the fulvic acids are lacking in long-chain alkyl carbons, and the hydrophobic portions of the molecules (aromatic rings) are highly oxygenated (each is a substituted phenol). The fulvic acid molecules are also structurally labile, as described above for the humic acids, and the abundance of polar and ionizable functional moieties account for their solubility in both acidic and alkaline solutions.

The humic acid structural moieties will also interact intramolecularly through hydrogen bonding, metal complexation, and other electrostatic interactions (discussed in Chapter 5). Combined, these inter- and intramolecular interactions stabilize (minimize) the electrostatic energy of the humic acid molecule. However, this structure is not static; indeed, the molecular configuration of a humic acid molecule is flexible (labile) and a function of the salt concentration and pH of the aqueous environment. High aqueous salt concentrations or low pH values collapse the flexible, random coils into globular aggregates or ring-like structures (Figure 4.33); whereas, low aqueous salt concentrations and high pH conditions expand the flexible coils to form thread- or net-like structures. The humic acid pseudostructure in Figure 4.30 also contains numerous nonpolar aromatic units and alkyl carbon chains, illustrating the amphophilic character of the substance. Because these structures are hydrophobic, they tend to aggregate in the interior of the humic acid molecule and weakly interact with one another via van der Waals forces. These nonpolar structural components are shielded from the aqueous solution by the polar and ionic portions of the molecule, which tend to form an external shell (akin to a micelle structure).

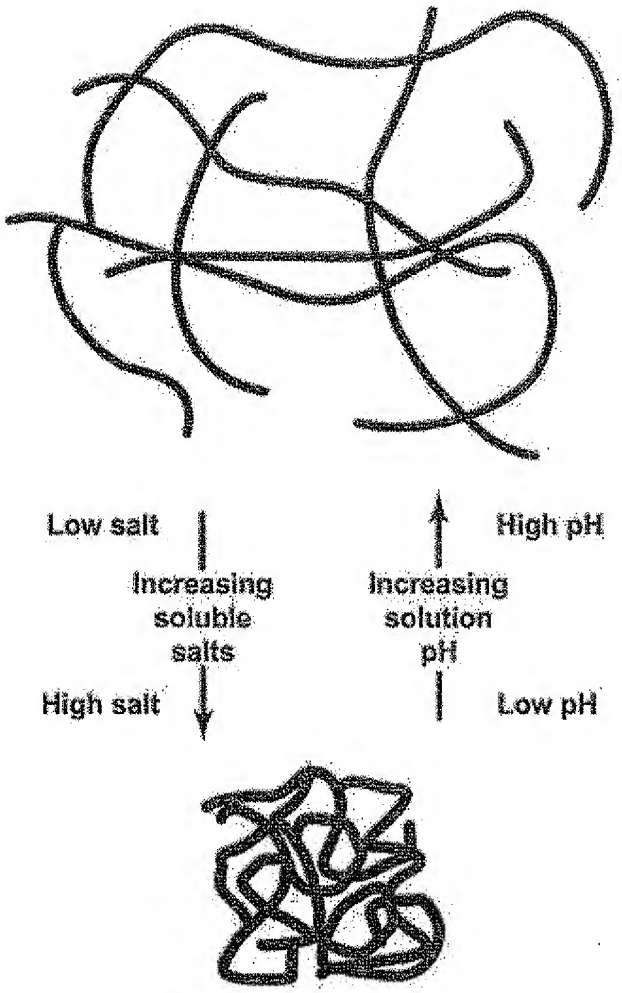


FIGURE 4.33 The molecular configuration of the flexible, random coils of the humic acid molecule as a function of the salt concentration and pH of the aqueous environment.

4.5 EXERCISES

1. *Polyfunctionality* and *structurally labile* are terms often used to describe the structural characteristics of humic substances. Define these terms.
2. Describe how the structural characteristics of polyfunctionality and structural lability combine to impart *hydrophilicity* to humic substances in aqueous environments.
3. In your own words, develop definitions for:
   a. Humic substances
   b. Humic acids
   c. Fulvic acids
   d. Humin
4. Compare and contrast the pathways that have been proposed to describe the formation of humic substances (see Figures 4.22 and 4.24).
5. A 1.0-g surface soil sample is digested with 5 mL of a standard 0.167 *M* $K_2Cr_2O_7$ solution and 7.5 mL of concentrated H_2SO_4 according to the Walkley-Black procedure. Titration of the digestate to the *N*-phenylanthranilic acid end point requires 3.11 mL of a standard 0.2 *M* $Fe(NH_4)_2(SO_4)_2$ solution. Compute the SOC content of the soil sample.

6. A soil humic acid is found to have O/C and H/C of 0.50 and 1.00. Answer the following:
   a. What is the average chemical formula of the humic acid?
   b. Write the reaction that describes the complete oxidation of this humic acid by $K_2Cr_2O_7$ to form Cr^{3+} and CO_2 (as done for CH_2O in Equation 4.1).
   c. Equation 4.1 indicates that 1 mol of $Cr_2O_7^{2-}$ consumes 1.5 mol of SOC. Is this assumption valid for the reaction generated in part 6(b) above?
7. Lignin theory describes the least humified humic substances as humins and the most humified substances as fulvic acids. The polyphenol theory, however, states that fulvic acids are the initial products of humification and humin is the most humified substance. Which of these two pathways is supported by the elemental content data presented in Table 4.3 (use the "All sources" data for each humic substance in your evaluation)?
8. Comment on the statement: "humic acids have lower total acidity and carboxyl contents and greater aromaticity than fulvic acids. The molecular masses of fulvic acids are also much less than those of humic acids. Therefore, humic acids may be isolated by acidifying an alkaline soil extract."
9. The elemental composition of the humic and fulvic acid fractions of an Elliott soil (an Aquic Argiudoll) is given in the table below. Answer the following:
   a. Calculate the molar O/C and H/C of the humic and fulvic acids and compare your results to the O/C and H/C of acetic acid (a simple aliphatic acid) and benzoic acid (a simple aromatic acid). Discuss your findings.
   b. Calculate the maximum potential negative charge that can be created on the fulvic and humic acids, assuming that the oxygen content is assigned to carboxyl groups. Express your results in $cmol_c\ kg^{-1}$. Compare your results to the cation exchange capacity values attributed to smectites and vermiculites.
   c. Calculate the maximum potential positive charge that can be created on the fulvic and humic acids, assuming that the nitrogen content is assigned to amino groups. Express your results in $cmol_c\ kg^{-1}$.

Elemental Content (in g kg^{-1}) of Humic and Fulvic Acids Extracted from an Elliott Soil

Compound	C	H	O	N	S
Humic acid	581	36.8	341	41.4	4.4
Fulvic acid	506	37.7	437	27.2	5.6

Data from the International Humic Substances Society.

10. An antiquated method for determining the carbonate and bicarbonate concentrations in soil solutions involves titration with standardized sulfuric acid, first to the phenolphthalein end point, then to the methyl orange endpoint. It is commonly observed during the titration of soil extracts that a brown precipitate forms in the titrand as the titration approaches the methyl orange endpoint. Describe the chemical process occurring in the titrand.
11. A pH 4.8 soil solution contains 0.05 mmol L^{-1} of succinic acid, a dicarboxylic acid that dissociates according to the following reactions:

$$HOOCC_2H_4COOH \rightarrow HOOCC_2H_4COO^- + H^+ \ (pK_{a1} = 4.21)$$

$$HOOCC_2H_4COO^- \rightarrow {}^-OOCC_2H_4COO^- + H^+ \ (pK_{a2} = 5.64)$$

What are the concentrations of $HOOCC_2H_4COOH$, $HOOCC_2H_4COO^-$, and $^-OOCC_2H_4COO^-$ in the soil solution?

12. A pH 5.2 soil solution contains 0.5 mmol L^{-1} of acetic acid, a monocarboxylic acid that dissociates according to the following reactions:

$$CH_3COOH \rightarrow CH_3COO^- + H^+ \ (pK_{at} = 4.76)$$

What are the concentrations of CH_3COOH and CH_3COO^- in the soil solution?

13. Comment on this statement: "molecular models that illustrate the structure of humic or fulvic acids are cartoons."

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EXHIBIT C

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THE PRINCIPLES OF HUMIC SUBSTANCES

Patrick MacCarthy

Two principles are presented that define the molecular nature and ecological role of humic substances (HS). The First Principle (i) accounts for and organizes an extensive body of apparently disparate data relating to the inability to purify and establish a molecular structure for HS; (ii) offers a conceptual framework for dealing with HS and for evaluating the applicability and limitations of various experimental methods; and (iii) identifies molecular heterogeneity, in combination with pronounced chemical reactivity, as constituting the essence of HS. Five corollaries to the First Principle spell out its consequences in more specific detail. New definitions of HS that offer greater insight into the molecular nature of these materials arise from the First Principle. The inapplicability of the molecular structure concept to HS is explained. The concept of hypothetical pseudostructures is introduced to help visualize the chemical reactions and interactions of HS without the unjustified assignment of specific structures to the material as a whole. Constraints in the design of experiments and in the interpretation of experimental data caused by the heterogeneous nature of HS are discussed. The Second Principle makes a connection between the molecularly heterogeneous and chemically reactive nature of HS and the ecological need for a reactive and persistent medium for plant growth. Concepts presented herein have broad implications in many fields, including chemistry, geochemistry, environmental and soil sciences, and ecology. (Soil Science 2001;166:738–751)

Key words: Humic substances, humic acid, fulvic acid, humin, humus, principles of humic substances, pseudostructures, supermixture, ecology.

THE presence of humic substances (HS) in the environment has long been recognized (Kononova, 1966; Schnitzer and Khan, 1972; Orlov, 1985; Frimmel and Christman, 1988). The term humic substances refers to a category of naturally occurring materials found in, or extracted from, soils, sediments, and natural waters. They result from the decomposition of plant and animal residues. Humic substances are found in all terrestrial and aquatic environments (Gjessing, 1976; Thurman, 1985) and constitute one of the most abundant forms of organic matter (OM) on the surface of the earth (Woodwell and Houghton, 1977; Woodwell et al., 1978). To some extent, these materials are defined by default – they are a category of natural substance that cannot be classified into any of the normal, easily defined categories of discrete materials such as proteins, polysaccharides, and polynucleotides.

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A BRIEF OVERVIEW OF HUMIC SUBSTANCES

Conventional Definitions and Terminology of Humic Substances

Humic substances are conventionally defined as "a series of relatively high-MW, brown to black colored substances, formed by secondary synthesis reactions" (Stevenson, 1982) or as "a category of naturally occurring, biogenic, heterogeneous organic substances that can generally be characterized as being yellow-to-black in color, of high molecular weight (MW), and refractory" (Aiken et al., 1985, p. 4). These vague definitions teach little about the chemical nature of humic materials. Because of the vagueness of these and other prevailing definitions of HS, it is common to also de-

fine these materials operationally in terms of the methods used to extract or isolate them from soils, sediments, and natural waters. The classic soil extraction procedure yields three main fractions: humic acid (HA), fulvic acid (FA), and humin. These fractions are defined operationally in terms of their solubility in aqueous media as a function of pH or in terms of their extractability from soils or sediments as a function of the pH of the extracting medium. *Humic acid* is the fraction of HS that is not soluble in water under acidic conditions, but becomes soluble (or extractable) at higher pH values. *Fulvic acid* is the fraction that is soluble in aqueous media at all pH values. *Humin* represents the fraction that is not soluble in an aqueous medium (or is not extractable with an aqueous medium) at any pH value. Actually, humin consists of an aggregate of humic and nonhumic materials (Rice and MacCarthy, 1990); as such, humin is better described as a humic-containing material rather than as a humic substance. The adjective humic is commonly used in a generic way to refer to each of these fractions. The term *humus* is often used synonymously with HS, but in other cases humus is used to include both humic and nonhumic material (Stevenson, 1982). It is assumed that free, identifiable constituents such as amino acids, sugars, and polysaccharides, which are co-isolated with the humic material, are removed before the extracted materials are considered to be exclusively humic as distinct from humic-containing. Complete segregations of this type are more readily hoped for than accomplished, as evident from the following two sections.

The Humic Substance-Nonhumic Substance Boundary

Humic and nonhumic substances share the same types of functional groups and other chemical characteristics. Consequently, it is a challenge to devise experimental methods that segregate these two classes of materials from each other in an absolutely definitive manner. As a result, it is difficult to confirm that a material that is considered to be a humic substance does not have some nonhumic substances mixed in with it. This situation is more likely to be encountered with FA and other lower MW fractions. Similarly, it is a challenge to identify the point in the decay process at which the transition from nonhumic to HS has occurred. Such uncertainties appear to be endemic to the field of HS and contribute to what has classically been referred to as "the humic acid problem". These complexities underlie the difficulty in defining HS, as evident throughout this paper.

Extraction/Isolation of Humic Substances

Humic substances are generally extracted from soil and sediment samples by treating the substrate with a basic solution (Stevenson, 1982; Hayes, 1985). Humic acids and FAs are co-extracted into this solution, and the unextracted residue contains the humin. When the alkaline extract is acidified by addition of a strong acid such as HCl, a material precipitates that is defined as HA, and the remaining organic material in solution is referred to as the fulvic acid fraction (Stevenson, 1985). Further steps are then taken to wash the HA free of other materials, to separate the FA *per se* from the other materials in the fulvic acid fraction, to diminish the ash contents of the humic and fulvic extracts, and to fully convert the fractions to their hydrogen forms. Part of the predicament in dealing with these elusive substances, as mentioned above, is that there is no definitive method for absolutely separating all nonhumic material from HS. Consequently, some pragmatic compromises must always be made in the extraction/isolation of HS. Some chemical degradation occurs during the extraction of HS. Thus, base-extracted HS generally comprise a combination of native and altered materials. The extracted HS are frequently dried by conventional evaporation or by lyophilization. It is also likely that some chemical changes, such as the formation of anhydrides and lactones and/or loss of carbon dioxide, occur when HS are dried, particularly when dried at elevated temperatures.

There are numerous variations of the extraction procedure including: the nature and concentration of the extractant used; the temperature at which the extraction is performed; period of contact with base; steps taken to minimize ash content of extracted products; and the choice of aerobic versus anaerobic conditions during the extraction. Many other extraction procedures and variations have been used, some involving various organic solvents such as dimethylsulfoxide, dimethylformamide, and formic acid (Hayes, 1985). It is not surprising that materials extracted from soils or sediments according to procedures based on the above definitions actually consist of mixtures; however, the degree of complexity found in these mixtures is profound. The singular designations for HA and FA are clearly generic terms representing mixtures of diverse molecules. Variations on the above procedure are used to isolate aquatic HS from natural waters (Riley and Taylor, 1969; Thurman and Malcolm, 1981; Aiken, 1985; Thurman, 1985; Serkiz and Perdue, 1990).

Properties of Humic Substances

All HS are amorphous and consist of complex mixtures. No study has come close to isolating a significant amount of any material that could be referred to as a pure or nearly pure HS. Therefore, most data on HS refer to average properties of a large ensemble of diverse molecules. The precise properties of a given humic extract may depend on the particular substrate chosen and the specific conditions of extraction. Nevertheless, there is a remarkable uniformity in the average properties of all HAs, FAs, and humins (Schnitzer, 1977; Rice and MacCarthy, 1991).

Elemental contents of HAs, FAs, and humins from all over the world are remarkably consistent (Rice and MacCarthy, 1991). Humic acids have been reported to have average MWs varying from about 2000 Da for aquatic materials to greater than 1×10^6 Da for soil-derived materials (Aiken and Wershaw, 1985), and some FAs have a number average MW in the range of about 600 to 900 Da. Humic substances have an abundance of oxygen-containing functional groups (carboxyl, phenolic, alcoholic) which dominate their chemical properties. In nature, and in the laboratory, HS constitute a bed of chemical reactivity or potential reactivity. While HS are often described as polymeric, a more appropriate characterization (at least for the larger molecular constituents) would be polyelectrolytic or macromolecular (Hayes et al., 1989, Ch. 1). It has not been possible to identify a unique molecular structure or a repeating structural unit in HS (Hayes et al., 1989) and, as stated by Gjessing (1976), "Humus is obviously not a definable organic compound, and it is unlikely that the composition will be clarified within the foreseeable future."

Soil Fertility, Environmental, and Geochemical Functions of Humic Substances

Humic substances participate in many agronomic, environmental, and geochemical processes (Hayes and Swift, 1978; Stevenson, 1982). For example, HS can serve as a reservoir for holding micronutrients in the soil and making them available later to plant root hairs. These materials also contribute to the acid-base buffering ability of soils, are able to bind mineral particles together in the soil environment, thus contributing to the structure of soil, and help to maintain the water regime of a soil. Other geochemical and environmental processes in which HS participate are dissolution of minerals (Hoch et al., 2000), binding of small organic molecules (Chiou et al., 1986), reduction of metal ions (Szilagyi, 1971), and mediating as an electron shunt in microbial and abiotic redox reactions (Wolfe and Macalady, 1992; Lovley et al., 1996). The variety and extent of these reactions and interactions indicate the highly reactive nature of HS.

Humic substances occur in close association with other organic and inorganic materials in soil and sediments. The humic and organic nonhumic materials are generally referred to collectively as OM, soil organic matter (SOM), or natural organic matter (NOM). These systems are not simple mixtures but involve multiple interactions and states of aggregation. Within this complex medium there is often an active microbial community. One may argue about the rationality of performing laboratory studies on a group of substances that have been segregated from the other living and nonliving entities in the environment, compared with studying the intact organic/inorganic composites in the presence of microbial communities. However, it seems that such laboratory investigations on isolated humic fractions provide the best hope for unraveling some of the fundamental chemical mysteries regarding the nature of these materials. Aquatic HS also occur in association with nonhumic materials and may exist in colloidal or larger aggregate forms.

OBJECTIVES

A vast body of empirical data has accumulated on the nature and properties of HS. What is lacking is an overall set of rules or guiding principles that account for the chemical nature and environmental role of these substances and which would serve as a conceptual model for further research on humic materials. The objective of this paper is to examine critically the extensive body of published data, prior observations, and ideas on HS in order to identify those features that are intrinsic and unique to humic materials. The results of this endeavor are expressed in the form of two principles. It is hoped that the principles espoused and opinions expressed in this paper will stimulate further examination of HS in an effort to substantiate the stated principles, to further refine them, or to invalidate them in full or in part.

THE PRINCIPLES OF HUMIC SUBSTANCES

The chemical nature and ecological role of HS can be rationalized on the basis of two fundamental principles:

I. *Humic substances comprise an extraordinarily complex, amorphous mixture of highly heterogeneous, chemically reactive yet refractory molecules, pro-*

duced during early diagenesis in the decay of biomatter, and formed ubiquitously in the environment via processes involving chemical reaction of species randomly chosen from a pool of diverse molecules and through random chemical alteration of precursor molecules.

II. *The molecular heterogeneity inherent in humic substances renders the humic material highly refractory, thereby serving a key role in the Earth's ecological system.*

The First Principle (MacCarthy, 2001) describes the fundamental molecular nature and origin of HS by addressing the questions: what are HS, and when, where, and how are they formed? The Second Principle, formulated previously as an hypothesis (MacCarthy and Rice, 1991), provides a connection between the molecularly heterogeneous constitution of HS and the ecological need for a medium that is both chemically reactive and refractory. These principles represent an attempt to look beyond the accidental and incidental in an effort to view the big picture and grasp the true essence of HS. The overriding truth of these broad principles supersedes the myriad details in the chemical and microbial processes that participate in the formation, reactions, and interactions of HS in nature and in the laboratory. Five corollaries are derived from the First Principle and reveal its consequences in more practical terms:

Corollary A. Humic substances are devoid of a regularly recurring, extended, skeletal entity.

Corollary B. Humic substances cannot be purified in the conventional meaning of purity.

Corollary C. The essence of humic substances resides in the combination of their extreme molecular heterogeneity and pronounced chemical reactivity.

Corollary D. Humic substances from different sources display a remarkable uniformity in their gross properties.

Corollary E. It is not possible to write a molecular structure or set of structures that fully describes the connectivity within molecules of a humic substance.

Corollary A states that there is no long-range chemical order recognizable in HS and that there is no identifiable backbone or skeletal structure that could be regarded as uniquely characteristic of these materials. Corollary B is self-explanatory. Corollary C recognizes those unique features that are essential to HS from both a chemical and an ecological point of view. Corollary D provides the basis for considering HS as a unique class of materials. Corollary E addresses the nebulous nature of the molecular structure concept when applied to HS. Data and arguments in support of the First Principle, and some consequences of the First Principle, are presented elsewhere (MacCarthy, 2001). Extensive data and arguments in support of the First Principle have also been presented in MacCarthy (2001) and will not be repeated here. In this paper, additional implications of the First Principle are presented, and the Second Principle is introduced and discussed in detail.

DEFINITIONS OF TERMS

The term *humic substances* is intended to designate that class of organic material occurring in or extracted from decayed or decaying biomatter in soil, sediment, or natural waters and that does not fall into any of the discrete classes of organic substances. Organic matter extracted from plants, senescent leaves, recently fallen leaves, and so on, is not considered to constitute HS in the context of this paper. The word *biomatter* refers to biologically synthesized matter that is no longer living or part of a living cell. The terms *heterogeneous* and *heterogeneity*, as used in the principles and throughout this paper, include the lack of structural regularity within and among the humic molecules; this topic is addressed more rigorously in a companion paper (MacCarthy, 2001). The term *regularly recurring, extended, skeletal entity* refers to a structural component that contains more than six C atoms and that occurs in an orderly, repeating manner within a molecule. *Chemical reactivity*, in the context of this paper, refers to the diverse reactions and interactions exhibited by HS in the environment. These reactions and interactions underlie the multiple functions of HS in nature and include: acid dissociation, metal complexation, ion exchange, sorption on minerals, and redox reactions with metal ions and organic species. The term *refractory* means that the substrate resists decomposition by micro-organisms, that is, microbial degradation of the HS is considerably slower than that of discrete biopolymers. It is not intended to imply that HS are absolutely recalcitrant and that they are not degraded by microorganisms.

THE FIRST PRINCIPLE—CONSEQUENCES AND IMPLICATIONS

Three consequences and implications of the First Principle have been presented and discussed elsewhere (MacCarthy, 2001):

(i) The combination of molecular heterogeneity and chemical reactivity constitutes the essence of humic substances
(ii) Humic substances constitute a distinct class of "natural product" that is unique and clearly different from the conventionally recognized natural products
(iii) Humic substances constitute a supermixture, i.e., a highly complex, heterogeneous mixture of molecularly diverse species in which the probability of finding two identical molecules is exceedingly small (excluding the very low MW fractions). Purification of a supermixture would, in the ultimate sense, involve a process approaching a molecule-by-molecule separation. This is the basis for Corollary B of the First Principle. Additional consequences of the First Principle follow.

New Definitions of Humic Substances

The First Principle actually constitutes a new definition of HS. This definition is molecularly based and is founded on a combination of chemical features (an enormously complex mixture of heterogeneous, chemically reactive, refractory molecules) and origin (ubiquitous substances formed via processes involving random selection of reacting species, early in the decay of biomatter). The new definition of HS does not obviate the need for the traditional, operational definitions that describe how humic fractions are actually obtained. Other definitions of HS could be formulated from the First Principle; for example:

(i) An unresolvable mixture of structurally heterogeneous reactive molecules formed early in the decay of biomatter
(ii) A naturally occurring mixture of organic molecules formed early in the decay of biomatter through processes involving molecularly nonspecific reactions, and
(iii) An even more cryptic definition: a chemically reactive supermixture extracted from decayed biomatter.

The Molecular Structure Dilemma for Humic Substances: In Search of the Nonexistent

Chemical Degradation Methods Applied to Humic Substances

What is meant by molecular structure in the context of HS? Application of classical structure-determining protocols to HS is severely constrained. The empirical formula for HS represents an average value and does not conform to simple integral atomic ratios (of course, all measured elemental contents can be forced into essentially integral values by imagining a sufficiently large MW). The measured MW of a given humic substance is also an average quantity (number average, weight average, or z-average). In the past, such elemental and MW average values have been combined to produce an "average molecular formula" for HS. Such average formulas, designed to conform to the average elemental contents and average MW of the sample, are no more meaningful than other hypothetical models that are not restricted rigidly to the measured elemental contents and average MWs. It is possible that either few or no molecules in the system display an elemental composition corresponding to the average values and that relatively few molecules in the mixture possess a MW equal to the average value. Similarly, the use of a "universal average formula unit" (Sein et al., 1999) for HA is not realistic.

Chemical degradation of HS produces a jumble of degradation products from the mixture of molecules in the system. It is not possible to identify which degradation products come from which parent molecule and, thus, there is no way to interpret such data rigorously in terms of structural formulas (or even molecular formulas) as can be done for pure compounds. It becomes clear that HS cannot be represented rigorously by a single molecular formula. It was for this reason that we previously chose not "to express the integrated information [for HS] as structural formulae" (Hayes et al., 1989, p. 29) and that Stevenson (1982) stated "no single structural formula will suffice" for HS. These considerations constitute the basis for Corollary E and are addressed more fully in the following sections of this paper.

Mass Spectrometric Methods Applied to Humic Substances

The problems that beset the classical structure-determining method, as applied to HS, are also the bane of more modern structure-determining tools, such as mass spectrometry. This is a common situation in humic studies, where the underlying complexity hinders the interpretation of data of all types. In classical mass spectrometry of a pure substance, some of the vaporized molecules are fragmented, the fragments as well as the molecular ion are identified, and the information is then mentally reconstructed to generate a structural formula. Application of this approach to HS produces a plethora of fragments from the myriad of diverse molecules in the original sample. One cannot

know which fragments came from which molecule, and it is not possible to establish individual structures from the intermixed fragmentation data.

The application of low-resolution, soft-ionization mass spectrometry to a mixture can provide information on the distribution of MWs in the mixture (Brown and Rice, 2000). High-resolution, soft-ionization mass spectrometry can separate the molecules in a mixture on the basis of their molecular formulas. However, the pattern obtained may not always be representative of the full suite of constituents in the mixture because of possible selective ionization of particular constituents. Each peak in such a spectrum may correspond to several or many constitutional isomers that cannot be segregated by mass spectrometry. Accordingly, application of fragmentation mass spectrometry to the components in a single peak from the high-resolution, soft-ionization mass spectrum of a humic substance would include fragments from multiple diverse molecules. This situation would limit one's ability to interpret the data in terms of discrete structures. The extent of these complications in mass spectrometric studies has not been fully investigated.

The difficulties encountered in the chemical degradative and mass spectrometric investigations of HS do not result from limitations in the chemical and instrumental tools, but, rather, they are inevitable consequences of the heterogeneous nature of humic materials. Based on the supermixture character of HS, it is to be expected that peaks obtained in high resolution, soft ionization mass spectrometry of HS represent a mixture of constitutional isomers. Recently, multistage tandem mass spectrometry (MS^n) experiments have been applied to molecular ions isolated by soft ionization mass spectrometric methods (Leenheer et al., 2001; Plancque et al., 2001). Leenheer et al. (2001) present fragmentation patterns to account for proposed molecular structures in the low MW fraction of an FA, and Plancque et al. (2001) propose an actual molecular structure for FA.

Meaning of Molecular Structure in Systems of Increasing Heterogeneity

Most mixtures can be separated into pure or relatively pure compounds for which molecular structures can be obtained. In the early days of protein investigations, the material presented itself as a mysterious mass, and progress in its understanding was slow. As time advanced, methods were developed for the separation and purification of individual proteins, and ultimately some proteins were obtained in crystalline form. Following the isolation of pure or relatively pure proteins, progress was rapid. The ability to conduct experiments on a substrate consisting of a single compound allowed the results of chemical degradation and other experiments to be interpreted in a rigorous manner, eventually leading to the complete primary structures of many proteins. That scenario is typical of experiences in most areas of chemistry, where progress was slow until pure or essentially pure compounds were isolated.

Humic substances do not yield to this type of investigative rigor. Since supermixtures cannot be separated into pure materials (Corollary B of First Principle), "determining the molecular structure" of humic substances would ultimately mean determining the individual structure of each molecule in the system—something that is not feasible. There is no substantive meaning to the term "molecular structure of humic substances," and knowledge of all the individual structures within a humic system is beyond our reach.

In the absence of a molecular structure for HS, and in view of the multitude of different structures within such systems, we must develop the ability to communicate outside the realm of conventional chemical reasoning. This is a most difficult undertaking for chemists, who, because of their education and experience, are accustomed to thinking in terms of discrete molecular structures. So what are we to do? What is the best manner of communicating ideas about HS (or any supermixture) at the molecular level? This question underlies the major difficulties in trying to comprehend HS. Historically, there have been two primary approaches to dealing with this problem, as discussed in the next two sections.

The "Write-No-Structure" Approach

In this approach, a decision is made to refrain from formulating structural models. Instead, the reactions, interactions, and other behaviors of HS are described in terms of the chemical and physical information that is available without trying to assemble such data into a molecular structure. This strategy is consistent with the approach that we adopted in the 1989 monograph "Humic Substances II: In Search of Structure" (Hayes et al., 1989).

Published "Molecular Structures" of Humic Substances

The second, and more common, approach has been to formulate molecular structures in an at-

tempt to describe the observed properties and behavior of HS. Numerous model structures of HS have been proposed over the years (Kononova, 1966; Schnitzer and Khan, 1972; Stevenson, 1982, 1985; Orlov, 1985, Ch. 11; Leenheer, 1989; Schulten and Schnitzer, 1993). While most of these models can account for some of the observed properties of HS, they also display many differences in the distribution of functional groups and in the nature and arrangement of the structural moieties. The diversity among the proposed structures illustrates the varied opinions among researchers about structural aspects of HS. The intended meaning of such published structures has varied with the particular author(s). In some cases, the models were intended to be interpreted quite literally, and in other cases they were simply meant as a guide to represent the type of chemistry that HS display. Sometimes it is not clear how the authors intended their proposed structures to be interpreted. The unfortunate consequence in all cases of proposed structures is that once such structures are published, they are frequently interpreted literally and cited as actual structures, regardless of what the original author(s) intended.

Perhaps, one way to acquire a feeling for the inherent complexity of HS is to imagine a concoction prepared by mixing molecules corresponding to many of the published models of HS. Such a multidiverse molecule model of the humic mixture would incorporate the structural ideas of many researchers and would mimic some of the heterogeneity and molecular diversity that is inherent to HS. If carried out indefinitely, this thought experiment would lead to a supermixture.

Introducing the Concept of Pseudostructures

Much is known and speculated about the composition of HS — elemental contents, functional groups and their relative abundances, various structural moieties such as aromatic and aliphatic segments, and so on. It is not possible to assemble this information into a discrete molecular structure for HA or FA. However, with a proper understanding of the nuances of supermixtures, one can assemble such fragmented data into figurative representations that incorporate compositional characteristics and other chemical features that have been identified in HS and their degradation products. While such models should be consistent with the known elemental, functional group and other structural moiety character of the materials, it is pointless to try forcing such model structures to adhere rigorously to a measured elemental composition, average functional group content, or average MW of a humic substance. These models are not intended to constitute actual molecular structures but merely to account for observed net properties and behavior of HS. Such mental constructs, in the form introduced in this paper, will be referred to as pseudostructures. Pseudostructures are *not* molecular structures, nor are they average structures or average structural models. Major differences between molecular structures and pseudostructures are listed in Table 1. The right column of Table 1 lists the numerous limitations of pseudostructures relative to genuine molecular structures (left column).

Pseudostructures are defined as hypothetical molecular constructs having elemental, structural, and functional group features consistent with some or all the observed properties of a given mixture. Nevertheless, individual pseudostructures may depart considerably from the measured elemental compositions, average functional group contents, and average MWs of a humic substance. Their form may also be influenced by conjectured precursor molecules and formation pathways. Pseudostructures offer a means for visualizing and communicating the types of chemical interactions and properties that a humic substance is expected to exhibit, based on experimental data, rather than trying to imagine and communicate these ideas in a more abstract space. Pseudostructures can account for general compositional features of a humic mixture, such as carboxylic acid acidity, phenolic acidity, complexation ability, aromatic and aliphatic content, and the presence of ester, ether, and other linkages in the system. They can also account for many other properties of HS, such as redox character, free radical nature, the ability to act as a cement between clay particles and to sorb nonpolar solutes, the tendency to engage in hydrogen bonding, and IR and NMR spectra. However, pseudostructures do not provide detailed information on the structure of a molecule as a whole, nor do they indisputably yield juxtapositional information on all functional groups or other structural moieties. That is because there is no "molecule as a whole" for these systems, and concepts that are normally chemically meaningful and definite—such as molecular formula and molecular structure—are not applicable to HS *en masse*.

Several, or many, different pseudostructures may be proposed to represent the same or different properties of a given humic substance. Pseudostructures are not restricted to a particular "molecular formula." A given pseudostructure may be intended to represent only certain proper-

TABLE 1

Comparison of pseudostructures with molecular structures

Molecular Structure	Pseudostructure
• is real, and corresponds to an isolatable substance	• a hypothetical construct that does not correspond to an isolatable substance
• describes a single substance	• describes properties of a complex mixture
• represents all (identical) molecules of a given compound	• it is possible that a pseudostructure is not identical to any molecule in a system
• is independent of how substance is produced	• may be influenced by conjectured precursor molecules and formation pathways
• corresponds to a particular molecular formula	• is not restricted to a specific "molecular formula"
• a single molecular structure is unique for a particular compound	• many different pseudostructures may be proposed to represent the same system
• describes a complete molecule	• may be intended to represent all or just selected features of a system
• provides all connectivity within the molecule	• provides little definite information about extended connectivity
• may allow calculation of distances between pairs of atoms	• should not be used for calculating extended interatomic distances
• possesses a specific molecular weight	• need not conform to a specific molecular weight (including average MW)
• must conform closely to measured elemental composition	• need not conform rigorously to measured elemental composition
• must include all elements from elemental analysis	• a given pseudostructure might not include some "minor" elements
• must include all known structural features*	• a given pseudostructure need not include all measured structural features*
• provides detailed stereochemical information	• does not provide rigorous stereochemical information
• provides a basis for anticipating molecular conformations	• should not be used as a basis for calculating detailed molecular conformations

*Structural features include measured functional groups, linkages, structural components such as aromatic and aliphatic moieties, and unpaired electrons.

ties of a humic substance. It is not necessary that each pseudostructure include all of the "minor" elements, such as N, P, and S, that may appear in the elemental analysis data. Pseudostructures must be distinguished from molecular structures which are intended to be interpreted literally, and from other molecular models that are generally assumed to provide reasonable representations of the extended connectivity within specific molecules. It is likely that no molecule in a humic system is identical to a proposed pseudostructure.

The potential problem with using pseudostructures is that they resemble molecular structures and could easily be misinterpreted as such. A pseudostructure differs from a molecular structure not in its diagrammatic appearance on paper, but, rather, in the manner in which it is interpreted, as governed by the limitations outlined in Table 1. Since a pseudostructure comprises a set of atoms joined by chemical bonds, it can be distinguished from a molecular structure only by the context in which it is presented. It is suggested that each pseudostructure be labeled with the term PSEUDOSTRUCTURE to emphasize its hypothetical nature and severe limitations and to help guard against overinterpretation. It is the author's contention that all proposed molecular models of HS are, at best, pseudostructures, and are therefore subject to the limitations set forth in Table 1. The pronounced limitations of pseudostructures, evident from Table 1, are often ignored in the interpretation and use of proposed molecular models of HS.

Figure 1 shows four pseudostructures, although not originally described as such, adapted from published model structures for Suwannee River FA (Leenheer, 1989; Saleh, 1989; Leenheer et al., 1998). Pseudostructures (a) and (b) were devised to represent Suwannee River FA (M_n = 800 Da). Pseudostructures (c) and (d) were drawn to represent a special metal-complexing fraction (7.1%) of Suwannee River FA (M_n = 956 Da), thus also serving as pseudostructures for the FA itself. The structures in Fig. 1 were written to conform, as closely as possible to the measured elemental compositions, average functional group

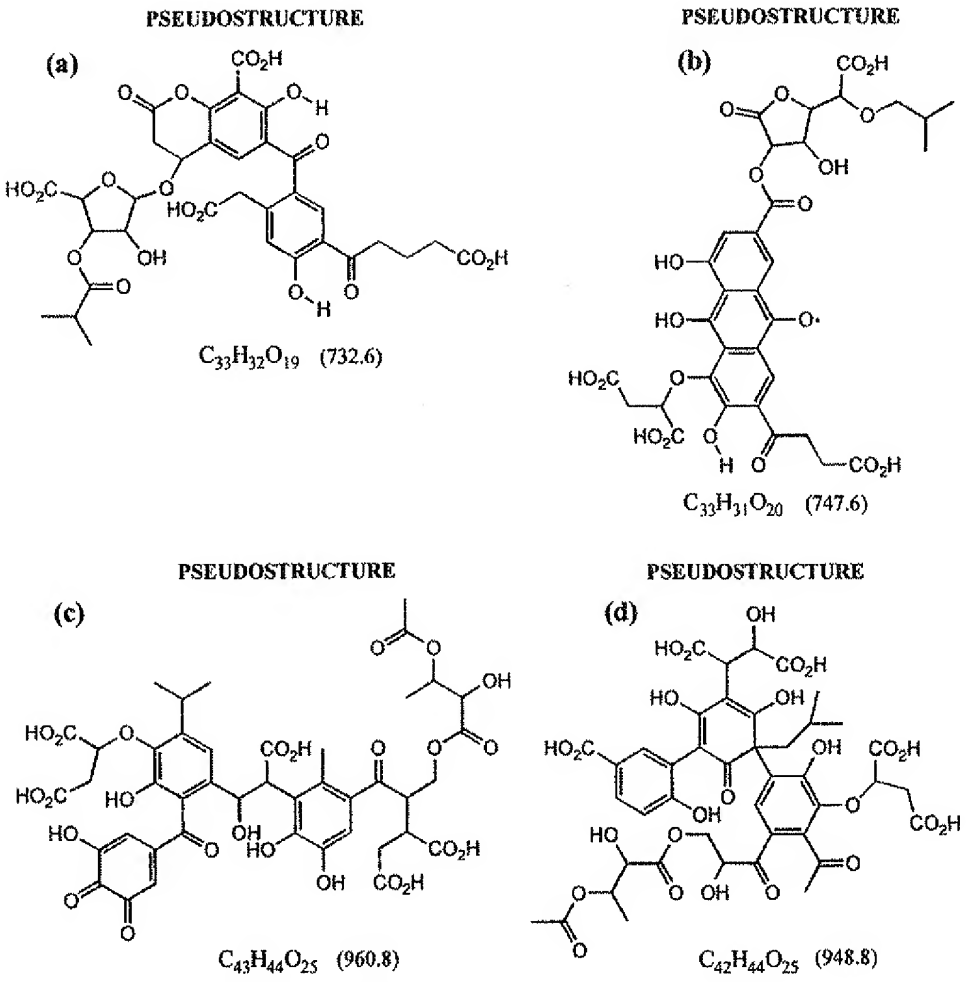

Fig. 1. Set of pseudostructures for Suwannee River fulvic acid.

compositions, and number average MW of the material (Leenheer, 1989; Leenheer et al., 1998). However, as noted above, such restrictions are not a requirement for pseudostructures, and many other valid pseudostructures that do not adhere to those constraints in composition and mass could be written for these materials. These structures were chosen as pseudostructures for this paper because of the extensive body of analytical data and critical thinking employed in their generation. The four structures in Fig. 1 were designed to reflect precursor materials from which the FA was speculated to originate (Leenheer, 1989; Leenheer et al., 1998). As evident from reading the original papers, these representations are not intended to depict actual molecules within the humic material, and "the assembly of structural data into a chemical structure [pseudostructure—added by author] is a subjective, speculative activity" (Leenheer et al., 1998). Rather, these figurative representations are hypothetical constructs intended to suggest types of chemical systems that would account for the reactions and other behavior of HS as observed in the laboratory and in the environment. Each pseudostructure in Fig. 1 is accompanied by its formula and the formula weight obtained by summing its atomic weights.

The pseudostructures in Fig. 1 display many similarities and differences, and each was designed to illustrate particular characteristics. They all consist of C, H and O only, and none of the "minor"

elements N, P, and S, is included, even though these three elements, collectively, comprise about 1.4% of Suwannee River FA by weight. Each of these pseudostructures has an abundance of carboxyl groups, and they all contain phenolic and alcoholic functional groups, ketone groups, aromatic and aliphatic moieties, and ester and ether linkages. All carboxyl groups in pseudostructure (c) are aliphatic whereas the other three pseudostructures contain both aliphatic and aromatic carboxyls (the aromatic carboxyl group in (b) is esterified). All aromatic rings in pseudostructure (b) are fused, whereas the other pseudostructures have no fused aromatic rings. Only pseudostructures (a) and (b) contain lactones, and only (d) possesses an enol functionality. Pseudostructure (b) contains an unpaired electron, and was written specifically to represent the free radical character of HS, even though it is estimated that Suwannee River FA contains only 1 to 2 free radicals per 1,000 molecules (Saleh, 1989). Pseudostructures (a), (b), (c), and (d) contain 5, 5, 7 and 7 asymmetric centers, respectively. The stereochemistry at each of those sites cannot be specified and, accordingly, one is not justified in presenting detailed stereochemical representations of HS. All four pseudostructures in Fig. 1 display ample opportunity for intramolecular hydrogen bonding and for chelation of metal ions. Other models that could be considered as pseudostructures have been designed to illustrate N-, P- and S-containing entities (Thurman and McKnight, 1989), the presence of fluorophores (Goldberg, 1989), and various metal-complexing sites (McKnight, 1989) in Suwannee River FA. The extended connectivity in all of these pseudostructures is speculative, and the particular form of a given pseudostructure reflects the bias of its creator(s). Thousands of other pseudostructures for Suwannee River FA could be constructed from the same data used to produce the drawings in Fig. 1. Pseudostructures of greater mass than those shown in Fig. 1 would afford more opportunity for depicting diversity and multiplicity (MacCarthy, 2001) within Suwannee River FA. The pseudostructures for Suwannee River FA presented here could also suffice for HA; in this case, additional pseudostructures could also be written, many considerably larger than those for FA.

On Molecular Conformations of Humic Substances

The extended connectivity shown in published molecular structures of HS is not justified beyond the level of speculation. Nevertheless, such structures are sometimes used as a license to interpret humic data with a degree of molecular detail that is unwarranted. Considering that there is no "molecular structure of HS" and that the stereochemical distribution around individual C atoms is not known, the issue of molecular conformations becomes even more elusive than that of primary structure. The use of computational programs for calculating discrete molecular conformations of HS or "building blocks" of HS (Sein et al., 1999) ignores the most fundamental feature of all HS—that they are indeterminate mixtures with no evidence for extended regularity in their structures. The very meaning of molecular conformation in this context is unclear. Despite the above restrictions, it is possible to experimentally obtain meaningful information on macromolecular properties of HS (Hayes et al., 1989, Chaps. 15–22).

Constraints in the Interpretation of Humic Data

The unyielding nature of HS to the conventional mode of chemical thinking and investigation has been illuminated throughout this paper, from the initial attempts at defining these materials to the nebulous meaning of their molecular structures. An examination of the humic literature reveals what appears to be a bifurcated comprehension of these materials. While there is general acknowledgment that HS comprise complex mixtures, that basic fact is often simply ignored in the design of experiments and in the interpretation of experimental data. That continues to be the most widespread flaw in the interpretation of humic data. An acceptance of the First Principle would help to remedy this situation.

Whereas in some cases one can provide satisfactory explanations in general terms for the actions of HS, it is often not possible to ascribe a definitive explanation to the observed effects. This is particularly true for experiments having a biological component, such as investigations of the influence of HS on plant growth (Burns et al., 1986; Chen and Aviad, 1990; Clapp et al., 2001) or human health (Klocking, 1994). Because of the unusual difficulty of interpreting data from HS, it is advisable that experiments on these materials be preceded by, or be conducted concurrently with, corresponding experiments using a temporary surrogate system in place of HS. Such surrogate systems may consist of discrete chemicals or contrived mixtures of discrete compounds. This approach allows one to investigate the applicability of the experimental methods and to evaluate the interpretability of the data in a manner that can be assessed more objectively before proceeding to the more complex humic system.

Recognizing Major Advances in Fundamental Humic Studies

A review of the history of HS research over the past 30 years reveals many interesting developments: the discovery that chlorination of humic-containing waters produces carcinogenic compounds (Bellar et al., 1974; Rook, 1974); advances in the isolation and treatment of HS through the use of various resins (Aiken, 1985); and increased knowledge of the composition of HS through the application of NMR and mass spectrometric methods. In addition, much has been learned about the macromolecular nature and micellar properties of these materials. Nevertheless, it may seem, at times, that there have been no major breakthroughs in the fundamental understanding of HS *per se* at the molecular level. This impression may persist even if the period of review is extended back 50 or 100 years! The reasons for this perception should now be evident. Some of the events that might have been considered breakthroughs, had they occurred, would have been: the isolation of pure fractions of HS, or the identification of dominant molecular structural units, or a molecular backbone for HS. Whereas these goals may have been long-term pursuits of researchers, it is now clear why they and other similar conceivable breakthroughs did not, and in fact could not, materialize. The very nature of HS precludes such eventualities.

In examining the history of humic studies it becomes evident that developments in this area did not occur in singular breakthrough events. Advances in this field have occurred through the long-term, steady accumulation of data and through sporadic observations described throughout the literature relating to the unusual nature of these materials. Actually, there have been significant advances in the understanding of HS, but because they have not satisfied the normal expectations of a breakthrough and because they did not lend themselves to discrete predictions, the advances have not been generally recognized as such. The actual advances are found in the scattered statements alluding to the fundamental nature of HS -- such as those of Dubach and Mehta (1963) about the possibility of no two molecules of HS being exactly alike, of Stevenson (1982) about the lack of molecular regularity in HS, and of Swaby and Ladd (1962) about the chaotic manner in which HS are formed. It is the ideas within those and other statements that have been compiled and expressed as part of the First Principle of humic substances.

THE SECOND PRINCIPLE—CONSEQUENCES AND IMPLICATIONS

Persistence-cum-Reactivity—the Ecological Uniqueness of Humic Substances

The Second Principle makes a connection between the molecular heterogeneity of HS and the fulfillment of an ecological need. It provides ecological significance to the molecular heterogeneity that is intrinsic to these ubiquitous materials. This principle teaches that HS are not just nature's junk resulting from decay but rather that they represent a life-sustaining force by virtue of their unique molecular constitution. It is instructive to compare the role of discrete molecules in a biological system with the role of HS in an ecological system. Biological molecules such as hemoglobin, chlorophyll, and enzymes are designed to engage in specific reactions and to perform unique, highly specialized tasks within living organisms. This high degree of specificity in biological systems requires the participation of molecules each having a unique molecular structure and formed from specific precursors through a definite pathway.

In contrast to the highly specialized and individualized roles of molecules in biological processes, the functions of HS in the environment do not necessitate the participation of specific molecules of uniquely defined molecular structures. The functions of HS in the soil environment (such as pH-buffering, binding of clay particles, serving as a reservoir for various micronutrient metal ions, sequestration and transport of metal ions, retaining moisture, etc.) are less specific than those in biological systems. In fact, the general functions of HS in the soil could, in principle, be satisfied by many of the direct, unaltered products of living cells such as proteins, polysaccharides, or polynucleotides. These biopolymers possess the requisite combination of hydrophilic, acidic, complexing and sorptive properties for performing the tasks cited above. Nevertheless, these biopolymers are not, and cannot, be directly utilized in place of HS in the ecological system.

Biopolymers generally do not survive for long periods in the open environment as they decompose rapidly through microbially mediated breakdown. In contrast, HS are refractory. According to Swaby and Ladd (1962), the resistance of HA to microbial and chemical decomposition could be explained if it consisted of "many heterogeneous units, irregularly cross-linked by different covalent bonds, so that innumerable extracellular enzymes from many different microorganisms would be needed to dismember it piece

by piece." Following up on the ideas of Swaby and Ladd, it was further proposed (MacCarthy and Rice, 1991) that HS, because of their structural heterogeneity, cannot serve as a template to guide the evolution of an organism with the ability to rapidly break down the material in the future. Thus, according to the Second Principle, *the molecular heterogeneity inherent in humic substances renders the humic material highly refractory, thereby serving a key role in the earth's ecological system.* This refractory nature confers a degree of environmental persistence on HS that is lacking in discrete biomolecules. Other refractory materials in nature lack the pronounced chemical reactivity exhibited by HS and, consequently, would not suffice in place of HS in the environment.

Several external factors have been proposed as contributing to the resistance of HS to breakdown by microorganisms. These factors include concealment of the humic material within cavities of a solid matrix that are too small to admit microorganisms, sorption of the HS on mineral surfaces, and complexation with metal ions (Swaby, 1968; Stevenson, 1982). Whereas such factors would also contribute to the preservation of discrete biopolymers such as proteins, the refractory nature of HS extends beyond what can be explained by those external influences and is manifested in the absence of any such external effects (Swaby and Ladd, 1962).

The molecular heterogeneity that is characteristic of HS serves a vital role in the ecological system. Humic substances constitute the only natural organic material that can survive in bulk and still possess the requisite chemical reactivity to perform the various functions for sustaining soil quality and promoting plant growth. In satisfying the need in soils for a persistent material capable of performing a number of functions necessary for plant growth, nature has adopted a simple and elegant solution whereby plants benefit vitally from the ubiquitous, immediately surrounding organic medium resulting from the decay of the plants' predecessors. By virtue of its molecular heterogeneity, this medium is highly biorefractory, but it still possesses the reactive functional groups needed to perform ecologically and environmentally vital tasks. The molecular heterogeneity residing within HS fulfills an ecological need in a unique way.

A NEW PARADIGM FOR STUDYING HUMIC SUBSTANCES

The inability to purify HS, the limitations of the molecular structure concept as applied to HS, and the subtlety of interpreting data from experimental measurements have all contributed to a bewilderment surrounding these materials. The confusion results from attempting to view HS from the same perspective as other classes of natural materials that are molecularly ordered, homogeneous or relatively homogeneous, and purifiable into discrete compounds. An acceptance of the principles presented herein causes the fog surrounding the understanding of HS to lift, and the features that previously seemed hazy now become quite clear. In fact, deviations from the actual observed behavior would now be considered a source of confusion! Accordingly, what is principally required for a fundamental understanding of HS are not just advances in separation techniques, instrumental methods, chemical degradation approaches, computational methods, or new methods of analysis but, rather, the acceptance of HS for what they really are, as embodied in the principles presented herein. In this approach, HS are consistently treated as supermixtures, and we are relegated to using crude pseudostructures in an attempt to communicate about the properties and interactions of these materials. An understanding of HS requires us to step away from the comfort of our normal chemical logic, where concepts of stoichiometry, discrete molecular structure, and purity prevail, and enter a more uncertain and challenging space that imposes constraints on our chemical reasoning and where the obvious is frequently far from evident!

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EXHIBIT D

MOLECULAR WEIGHT AND SHAPE OF HUMIC ACID FROM SEDIMENTATION AND DIFFUSION MEASUREMENTS ON FRACTIONATED EXTRACTS

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Summary

Whole humic acid extracts are usually too polydisperse for reliable molecular-weight measurement to be made in the ultracentrifuge by the sedimentation velocity technique. Consequently, the humic acid used in this study was fractionated with respect to molecular weight into fractions of low polydispersity by extensive use of gel-permeation chromatography and other fractionation techniques.

The sedimentation and diffusion coefficients of the fractions were determined and molecular-weight values calculated. These values ranged approximately from 2×10^3 to $1{\cdot}5 \times 10^6$, the higher figure not necessarily representing the upper limit for these substances.

On the basis of the frictional parameters calculated from the experimental data, it is proposed that the molecule adopts the solution conformation of a randomly coiled polymer in which branching may be significant, particularly at higher molecular weights.

Introduction

ACCURATE information on the molecular size and shape of humic acid would complement our present knowledge of its chemical and physical properties and aid our ability to understand its functions in the soil. The lack of really definitive values for the molecular weight of humic acids can largely be attributed to the extremely polydisperse nature of these substances. It is generally accepted (Dubach and Mehta, 1963) that humic substances extracted from surface soils have molecular weight values ranging widely from approximately one thousand to several hundred thousands with variously reported mean values of three to fifty thousands. In such a system, however, mean molecular weights are of limited value and the molecular-weight distribution is of more practical interest.

Several studies have been reported (Stevenson *et al.*, 1953; Piret *et al.*, 1960; Flaig and Beutelspacher, 1968) in which the analytical ultracentrifuge has been used to determine the molecular weights of humic acids, and in each case the sedimentation velocity technique was employed. In the application of this technique, however, the innate properties of humic acid present several problems which must be overcome. These problems arise from the intense colour of humic acid solutions, its highly charged polyelectrolyte nature and its extreme

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polydispersity. The effects of colour and charge can be overcome by suitable manipulation of the experimental operating conditions, but up to now no attempts have been made to solve the problems presented by polydispersity when measuring the molecular weight of humic acid by ultracentrifugation.

In the work presented here, humic acid extracts have been extensively fractionated on the basis of molecular size by means of gel-permeation chromatography into a series of fractions of varying molecular weight and greatly reduced polydispersity. The sedimentation velocities and diffusion coefficients of these fractions have been determined and their molecular weights and frictional properties have been computed. In this way, the molecular weight range, rather than a mean value, has been determined and information has also been obtained concerning the conformation of the humic acid molecule in solution.

Experimental

The soil used in this study was a highly humified organic muck soil (45 per cent organic matter) under natural lakeside vegetation sampled to a depth of 7·5 cm.

Extraction

The soil was extracted sequentially, in the absence of air, with 0·1M sodium pyrophosphate, 0·5M NaOH (20 °C), and 0·5M NaOH (60 °C) using procedures described in greater detail elsewhere (Posner, 1966; Posner *et al.*, 1968). The humic acids were recovered by precipitation with H_2SO_4 at pH 1 and purified by redissolving at pH 7 by the addition of NaOH, centrifuging to remove insoluble materials, reprecipitating at pH 1 and removal of the supernatant liquid. After repeating this process several times the humic acid precipitates were thoroughly dialyzed before freeze-drying. In this way samples low in organic and inorganic impurities (0·1–2·0 per cent) are obtained (Posner, 1966).

Molecular Weight Fractionation

First Stage. The majority of the fractions used were derived from the 20 °C NaOH extract. They were obtained by fractionation of the extract on a large, preparative, 12 per cent agar gel column (48 × 14 cm) (Swift *et al.*, 1970). The crude humic acid (12 g) was converted to the ammonium salt by dissolving in NH_4OH and freeze-drying the solution. The salt was divided into six equal portions, each of which was dissolved in 150 ml of a carbonate/bicarbonate buffer (0·02M $NaHCO_3$+0·05M KCl+0·001M EDTA; pH 8·5, ionic strength = 0·08). The column was packed in and eluted with the same buffer and the retained coloured material was collected as 50–60 × 200 ml fractions. The chromatograms given by the six separate runs, obtained by monitoring the optical density of the eluate at 400 nm, were superimposed and corresponding fractions were pooled. Selected pooled fractions, adjacent to each other along the chromatographic spectrum, were further combined to yield seven crude batches which together represented much of the range of molecular weights occupied by the original 20 °C NaOH extract. The

humic acid was precipitated from each batch by acidification to pH 1, and the resulting precipitate repeatedly washed till salt free, centrifuging after each wash in a Beckman Model L2 preparative ultracentrifuge at about 150,000 gravity units to avoid losses due to dispersion. The precipitate was dissolved in tris buffer A (414 ml M tris + 50 ml M HCl)/l, pH 9·0, ionic strength = 0·05 to make a solution of concentration of not more than 40 g/l. Here 'tris' stands for 2-amino-2 (hydroxymethyl)-propane-1,3-diol.

In addition to the seven different batches obtained as described above, two additional crude fractions, one high and one low molecular weight, were prepared by different procedures from the other humic acid extracts. This enabled an assessment to be made of the feasibility of other methods of fractionation on the basis of gross molecular weight and extended the study to humic acids extracted by other reagents.

The low molecular-weight fraction was obtained from the sodium pyrophosphate extract by using a membrane filter with a low molecular-weight cut-off. An aqueous solution of the ammonium salt of the humic acid (2 g in 100 ml) was ultra-filtered (5 × 50 per cent volume reductions) through a Diaflow XM-50 membrane (nominal molecular-weight cut-off 50,000). The solution of low molecular-weight material passing through the membrane (approx. 0·7 g) was concentrated by evaporation under reduced pressure and made up to the strength of tris buffer A before proceeding to the next stage of the fractionation.

The high molecular-weight fraction was obtained from the 60 °C NaOH extract by means of preparative ultracentrifugation. An aqueous solution of the ammonium salt of the humic acid (2 g in 100 ml) was subjected to 150,000 gravity units for 6·5 h in a Beckman Model L2 preparative ultracentrifuge. The sediment (approx. 0·8 g) was re-dispersed in tris buffer A before further fractionation. The reduction in polydispersity obtained by these two procedures was less than that achieved using the agar column but the effort involved was correspondingly lower.

Second Stage. The various fractions obtained as described above required additional fractionation before they could be used satisfactorily in the analytical ultracentrifuge. The general procedure adopted for further reductions of sample polydispersity was as follows.

The seven pooled fractions obtained from the large agar column were applied as 10 ml of solutions, containing up to 0·4 g humic acid (from optical density measurements), to a Sepharose 6B (Pharmacia Fine Chemicals, Uppsala) gel column (61 × 2·6 cm) that was packed in and eluted with tris buffer A. The retained material was collected as 5 ml fractions and a chromatogram determined by recording the optical density at 400 nm. The central portion of the peak was cut at a width equal to the base width of a haemoglobin peak, recovered by precipitation at pH 1, then redissolved in tris buffer and re-applied to the same column. The tail portions of the peak were generally discarded. The process was repeated three or four times, at which stage the peak widths of only the low molecular-weight batches had approached that for a

monodisperse protein (haemoglobin). Nevertheless, the final refractionation had, in all seven cases, achieved only slight reduction in peak width. Considering both this and the rapidly diminishing yield from the high molecular-weight batches, further refining was considered uneconomic. The central portions of the seven final peaks were labelled B1 to B7, in order of their subsequently determined molecular weights. Each was precipitated at pH 1 and redissolved in tris buffer B [(414 ml M tris + 70 ml M HCl)/l, pH 8·2, ionic strength = 0·07] to give solutions of about 2–5 g/l.

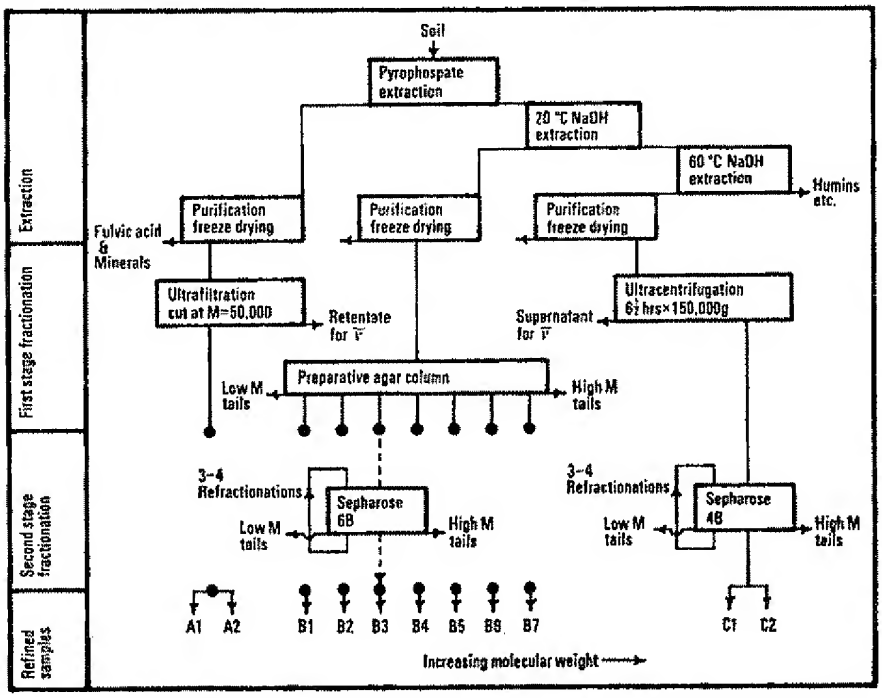

FIG. 1. Flow chart illustrating the preparation of refined humic acid fractions.

The success of the first few refractionations in narrowing the K_{AV} distributions of the peaks contrasted with the insensitivity of the fractions to further refractionation, indicating that although tris buffer greatly reduced gel/solute interaction with Sepharose gels, as proposed by Swift and Posner (1971), it did not completely eliminate it. The dispersion in final peak-retention volumes was therefore probably due at least as much to dispersion by gel/solute interaction as to polydispersity.

The two fractions obtained by preparative ultracentrifugation and membrane ultrafiltration were treated in a similar way except that the high molecular-weight material was fractionated on a Sepharose 4B column. Since these two fractions were quite polydisperse, a larger number of column runs was required before suitably narrow peaks were obtained. Two cuts on either side of the centre were taken from the

final peaks of both the low molecular-weight material (A1 and A2) and the high molecular-weight material (C1 and C2). The differing molecular weights ultimately found for each pair of off-centre cuts indicated considerable residual polydispersity in the peaks.

Retention volumes of all the refined fractions (A1, A2, B1–B7, C1, C2) were determined on various gel-chromatography media and appear in a later paper in this journal (Cameron *et al.*, 1972).

All samples derived from the second stage of this fractionation procedure were found to be suitable for use in the ultracentrifuge.

Fig. 1 summarizes the extraction and fractionation procedures described above.

Preparation of samples for ultracentrifugation and diffusion measurements

Before determining the sedimentation or diffusion coefficients of the fractionated humic acid samples, they were equilibrated with the buffer by dialysis. Samples (1–5 ml) at the desired concentration (from optical density measurements), in tris buffer B, were placed in Visking dialysis tubing and dialysed against 50 ml of the same buffer for 24–48 h. These external and internal solutions were used for the experimental determinations. When the lowest molecular-weight fractions were being processed dialysis was not possible but care was taken to maintain equal buffer concentrations. Microbial growth was minimized by performing dialysis at 4 °C and by passing humic acid solutions through a millipore filter immediately prior to the sedimentation and diffusion experiments which were conducted at 20 °C.

Ultracentrifugation

Sedimentation coefficients were determined using a Spinco Model E ultracentrifuge fitted with Schlieren optics for measurement of concentration gradients. The synthetic boundary technique was employed using a 4°, 12 mm synthetic boundary cell containing 0·4 ml of humic acid solution overlain by 0·2 ml of tris buffer B (equilibrium dialyzate). Because of the low concentrations of humic acid used, it was considered that ionic strength was sufficiently high for charge effects to be neglected. Failure to suppress charge effects leads to an increased rate of Schlieren-peak spreading, due to intermolecular charge repulsion, and the creation of an electric field opposing sedimentation, due to the different sedimentation rates of macromolecules and counterions. Humic acid concentrations were generally 1·2 g/l but varied in some runs between 0·6 g/l (below which the Schlieren peak became ill-defined) and 2·4 g/l (above which the light transmission was too low). Illumination was by means of a mercury lamp fitted with a filter to remove all but the red light and photographs were recorded on 35 mm Kodak 2475 recording film (1000 ASA). Exposures of 4 sec were made at intervals of 2 or 4 min over total run times of 20–40 min. A rotor speed of 59 780 rpm. was used for all but the very high molecular-weight fractions when a speed of 29 500 rpm. was employed. The movement of the Schlieren-peak maxima with time was measured by projection of the photographs on to graph paper.

The sedimentation coefficients calculated from these measurements increasingly underestimated the peak-median values, as profiles became more positively skewed with higher sample molecular weights. This was thought to be partly because higher molecular-weight fractions were more polydisperse, and partly because their Schlieren patterns were far less influenced by the diffusion effects which oppose positive skew in low molecular-weight profiles.

Diffusion measurements

Diffusion coefficients were determined by a modified free boundary method developed especially for the conditions encountered in this work and utilizing in particular the intense colour of the humic acid solutions. A sharp boundary was formed by carefully layering clear buffer on to dilute humic acid solution (approx. 0·1 g/l) in vertical, narrow bore (3 mm diam.) glass tubes using a small syringe. The formation of a sharp boundary was facilitated by having 30 per cent D_2O in the lower layer. The D_2O was added to aid the initial formation of a sharp boundary and to provide a density gradient within the tube capable of stabilizing the system against disruptive thermal effects which are likely to occur in experiments lasting several days, particularly for higher molecular-weight fractions. A small viscosity correction was made to allow for the slightly reduced diffusion rate in the presence of the D_2O gradient.

Measurements were made at approximately 20 °C and the concentration of the diffusing humic acid was monitored by scanning the transmission of light throughout the length of the tube at various intervals of time using a Densicord scanning densitometer fitted with a blue light filter. The calculation of the diffusion coefficient from the interquartile distance on the concentration gradient was shown numerically to yield approximately a weight-median value for a polydisperse system. Usually, several replicates were examined simultaneously. The method is more accurate than that in which diffusion values are determined in the ultracentrifuge at low speeds by measuring the rate of broadening of the Schlieren peak. The latter was found to be unsuitable for a substance which is not completely monodisperse. Fuller details of the method will be published elsewhere.

Partial Specific Volume

Two samples having similar mean molecular weights (around 100,000) were examined. One was the high molecular-weight material retained after ultrafiltration of the pyrophosphate extracted material and the other was the low molecular-weight material (supernatant) remaining after ultracentrifugation of the 60 °C NaOH extract, the smallest molecules being removed by dialysis. The tris salt of each was prepared and duplicate solutions of known concentration in tris buffer B made up. Using a normal pycnometer method, partial specific volumes of tris humate were determined by diluting the samples with the same buffer through the concentration range 25 to 0 g/l.

Calculations

The various parameters were calculated from the following relationships:

Sedimentation coefficients $$s = \frac{1}{\omega^2}\frac{d\ln x}{dt},$$

where x = distance of the Schlieren peak maximum from axis of rotation, ω = angular velocity and t = time. This value was adjusted to standard conditions (water at 20 °C) using:

$$s_{20} = \frac{s\eta_{T,\text{buffer}}(1-\bar{\nu}\rho_{20,H_2O})}{\eta_{20,H_2O}(1-\bar{\nu}\rho_{T,\text{buffer}})},$$

where η = viscosity, $\bar{\nu}$ = partial specific volume, ρ = density, T = temperature. The buffer viscosity was found to equal that of water.

Diffusion coefficients $$D = \frac{0{\cdot}2749\,d^2}{t},$$

where d is the distance between points taken at 25 per cent and 75 per cent of the concentration gradient. Diffusion values were corrected to standard conditions (water at 20 °C) using:

$$D_{20} = \frac{D_T\eta_T}{\eta_{20}}\cdot\frac{293}{T}.$$

Partial specific volume ($\bar{\nu}$)

$$(1-\bar{\nu}\rho) = \frac{1-W}{m}\cdot\frac{\partial m}{\partial W},$$

where $\bar{\nu}$ is the partial specific volume of the humic acid, ρ the density of the solution, m the mass of the pycnometer contents and W the weight fraction of humic acid.

Molecular weight

$$M = \frac{RTs}{(1-\bar{\nu}\rho)D} \quad \text{(Svedberg equation)}$$

where R = gas constant.

Intrinsic frictional coefficient

$$[f] = \frac{f}{\eta} = \frac{kT}{\eta D},$$

where f = frictional coefficient and k = Boltzmann constant.

Frictional ratio $$\frac{f}{f_{\min}} = \frac{1}{3\eta}\left(\frac{k^2T^2}{9\pi^2}\cdot\frac{(1-\bar{\nu}\rho)}{\bar{\nu}}\right)^{\frac{1}{3}}\cdot\frac{1}{s^{\frac{1}{2}}D^{\frac{1}{2}}},$$

where $f_{\min}$ is the frictional coefficient that the same molecule would have if condensed to a solid sphere.

Results and discussion

Ideally sedimentation velocity should be determined by extrapolation to zero concentration or by working at very low solute levels. The

latter is feasible for humic acid if UV-absorption optics are available, but the Schlieren optical system imposes its own, somewhat higher, concentration limit. Even so, it is thought that the solutions used here were sufficiently dilute to be near ideal behaviour. In addition it has been reported that humic acid sedimentation velocities are largely independent of concentration (Piret *et al.*, 1960; Stevenson *et al.*, 1953).

Charge interactions were minimized by working at a reasonably high ionic strength. Experiments performed with polyelectrolytes without suppression of charge effects give misleadingly high diffusion rates and low sedimentation velocities and such results do not give valid molecular weights. Secondary salt effects (Pedersen, 1958) and specific binding of counter ions (Huizenga *et al.*, 1950*a*, *b*) can influence the molecular weight obtained but these effects are estimated to be small.

The most difficult problem in the study of humic acid by ultracentrifugation is presented by its polydispersity (Flaig and Beutelspacher, 1968; Stevenson *et al.*, 1953). This characteristic impairs the formation of a well defined, sedimenting concentration boundary because the multifariously sized species will be sedimenting at different velocities. Such polydisperse systems give rise to highly asymmetric Schlieren patterns. Measurements made from the maximum of this curve would give a sedimentation coefficient which is not representative of the whole sample. If mean molecular-weight values are required for polydisperse extracts, a better way of obtaining them would be by the use of the sedimentation equilibrium technique rather than the sedimentation velocity technique (Posner and Creeth, 1972). The equilibrium technique requires a higher standard of machine performance, more sophisticated optics, and considerably longer running times than the sedimentation velocity technique.

Preliminary ultracentrifugation experiments with whole humic acid extracts quickly established that, because of their high degree of polydispersity, a Schlieren peak could not be maintained for any reasonable length of time despite the use of the synthetic boundary technique. In the first stage of the fractionation procedure, using preparative gelpermeation chromatography, a single extract was subdivided into approximately 50 fractions. Several of these, selected to cover the whole molecular-weight range, were again examined in the ultracentrifuge. Despite the large reduction in the molecular-weight spread of these fractions, giving some improvement in Schlieren-peak stability, their polydispersity was still too great for satisfactory use in the ultracentrifuge. Consequently, these first-stage fractions were further separated in a second stage by repeated gel-permeation chromatography with column and collection parameters chosen so as to improve resolution. In addition, the system was chosen so as to be as free as possible from gel/solute interactions (Swift and Posner, 1971) which interfere with molecular-weight fractionation.

The extent of narrowing of the elution peak obtained at various stages of the fractionation compared with the original extract is illustrated in Fig. 2. Because of peak spreading effects, the reduction in polydispersity is much greater than might be deduced by comparing the

widths of the final peaks with those obtained from earlier stages of the fractionation.

The final samples obtained by this process were found to be very satisfactory for use in the ultracentrifuge. Even so, they are still far from being monodisperse; the sedimenting Schlieren peak broadening

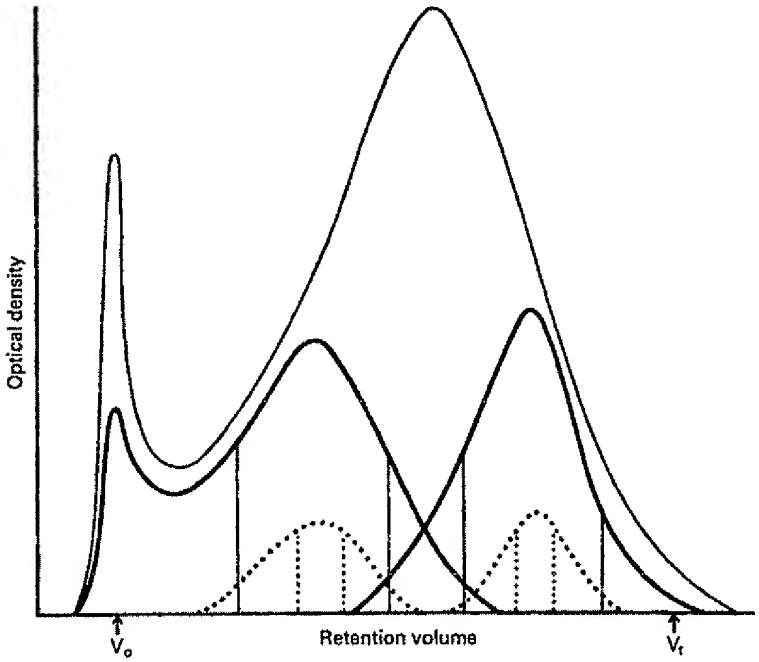

FIG. 2. Stages of refinement of two typical crude fractions obtained after eluting a whole humic acid extract on a preparative agar column. Each crude fraction was eluted on Sepharose 6B (——), and a central cut of width equal to the peak base width of haemoglobin was taken (i.e. the material between the solid vertical lines). This was re-run and again re-cut at the same position, the process being repeated 3–4 times. The final peaks obtained (·····) were cut at the width shown by dotted vertical lines, the central portions becoming the refined fractions used in the ultracentrifugation and diffusion experiments. The chromatogram of the whole parent extract on Sepharose 6B (——) is included to illustrate the distribution of material prior to fractionation on agar.

more quickly than would a monodisperse protein with a similar diffusion coefficient. In the light of these and other observations (Flaig and Beutelspacher, 1968; Stevenson *et al.*, 1953) the very stable peaks recorded by Piret *et al.* (1960) for a *whole* sodium hydroxide extract are remarkable.

Accurate diffusion coefficients are as important as sedimentation coefficients in the determination of reliable molecular weights. Gross polydispersity also adversely affects the determination of meaningful diffusion coefficients due to the different diffusion rates of the variously sized species. The measurements made on the fractionated samples should therefore be more useful than those previously reported.

Molecular Weights

Selected samples, representing the whole molecular-weight spectrum of humic acid, were taken and their sedimentation and diffusion coefficients in tris buffer determined. From these values and the partial specific volume, the corresponding molecular-weight values were calculated. Molecular weights derived in this way necessarily represent the aggregate molecular weights of all solute travelling within the domains of individual humate polyanions, i.e. they include a fraction of the protonated tris counterions (Huizenga *et al.*, 1950*a*, *b*).

TABLE 1

Molecular parameters of fractions

Fraction	*Extractant*	*Sedimentation** *Conc.* g l^{-1}	$s_{20} \times 10^{13}$ *sec.*	$D_{20} \times 10^{7}$ $cm^2\ sec^{-1}$	$M \times 10^{-3}$	$[f] \times 10^{5}$ *dyne* cm^{-1} *sec poise*$^{-1}$	$\frac{f}{f_{min}}$	$\frac{R_G}{M^{\frac{1}{3}}} \times 10^{9}$ cm	R_G Å
A 1	Na pyrophosphate	1·2	0·82	21·4	2·6	0·19	1·14	2·9	15
A 2	Na pyrophosphate	2·4	1·02	16·0	4·4	0·25	1·28	3·0	20
B 1	20 °C NaOH	1·2	1·88	10·2	12·8	0·40	1·41	2·8	32
B 2	20 °C NaOH	1·2	2·47	8·4	20·4	0·48	1·46	2·7	38
B 3	20 °C NaOH	1·2	2·64	7·7	23·8	0·52	1·52	2·7	42
B 4	20 °C NaOH	1·2	4·7	3·95	83	1·02	1·96	2·8	82
B 5	20 °C NaOH	1·2	5·6	3·07	127	1·32	2·18	2·9	105
B 6	20 °C NaOH	1·6	6·7	2·45	199	1·65	2·35	2·9	132
		0·8	7·2						
		0·6	7·2						
B 7	20 °C NaOH	1·2	12·6	2·13	412	1·90	2·12	2·4	153
C 1	60 °C NaOH	1·2	12·6	2·15	408	1·88	2·11	2·4	150
C 2	60 °C NaOH	2·4	24·6	1·26	1360	3·21	2·41	2·2	255

* Sedimentation coefficients obtained by a least squares linear fit had typical standard errors of 4 per cent for lower molecular-weight fractions, decreasing to 2 per cent at higher molecular weights.

Mean diffusion coefficients, obtained from 4 to 8 replicate runs, had standard errors between 1 to 5 per cent. Solution concentrations were approximately 0·1 g/l.

In this work, the partial specific volume used in calculations was taken as 0·65 cc/g. This was the mean of the experimental values of 0·63 cc/g (sodium pyrophosphate extract) and 0·67 cc/g (60 °C NaOH extract) for tris humate. The most meaningful partial specific volume would lie somewhere between the one used and that of the humate anion which was about 0·02 cc/g lower (assuming protonated tris constitutes 25 per cent wt. of tris humate and has $\bar{v} = 0{\cdot}715$ cc/g). Previously Stevenson *et al.* (1953) and Piret *et al.* (1960) have reported values of 0·67 cc/g and 0·71 cc/g respectively for humic acid. Posner and Creeth (1972) report a value of 0·64.

Calculated molecular weights probably understated weight-median molecular weights by a proportion which increased with their values. As noted in the experimental section, while diffusion coefficients represented the weight-median species for all samples, the measured sedimentation coefficients increasingly underestimated weight-median values for samples of higher molecular weight. This effect is considered to be relatively small (Cameron *et al.*, 1972).

The results obtained are shown in Table 1. The molecular-weight

values cover an extremely wide range, extending numerically from approximately $2{\cdot}6 \times 10^3$ to $1{\cdot}4 \times 10^6$. These findings supply more definite evidence for what has previously been inferred from gel-chromatography data concerning the humic acid molecular-weight range. The upper value is much higher than is usually quoted and there is little reason to assume that this is the real upper limit but rather a limit imposed by the difficulty in fractionating larger molecules on Sepharose columns. If this were overcome, solubility considerations and the extraction methods used would impose a further limit. The most abundant portion of the molecular-weight distribution of the cold NaOH extract occurs at a molecular-weight value of around 100,000 and the distribution is probably unimodal with an extended high molecular-weight tail.

Molecular Conformation

In general, the shape and behaviour of a polymer in solution can be approximated by one of several possible models (Tanford, 1961). The relationships between the frictional ratio (f/f_{min}) and molecular weight (M) for several common models are as follows:

(i) Solid hard sphere (i.e. no solvent trapped within the sphere) then, by definition,

$$\frac{f}{f_{min}} = 1{\cdot}0$$

and is independent of molecular weight.

(ii) Hard oblate spheroid (approximating to a roughly circular flat plate). If the mean molecular mass per unit area is assumed to be independent of molecular weight, then for high molecular weights and axial ratios $a/b > 50$,

$$\frac{f}{f_{min}} \propto M^{0{\cdot}165}$$

(at lower axial ratios the value of f/f_{min} is greater than that predicted by the above relationship). The relationship is unaffected by varying solvation.

(iii) Hard prolate spheroid (approximating to a stiff rod-shaped helical coil). If the mean molecular mass per unit length is assumed to be independent of M and the axial ratio $a/b > 50$, then

$$\frac{f}{f_{min}} \propto M^{0{\cdot}47}$$

(at lower axial ratios the value of f/f_{min} is greater than that predicted by the above relationship). The relationship is unaffected by varying solvation.

(iv) Flexible random coil. In the treatment used here, the frictional properties are considered to arise from a series of Stokes' spheres placed equidistantly along the molecular chain (Pearl necklace model[1] of Kirkwood and Riseman, 1958).

[1] The pearl necklace concept is a convenient device for ascribing frictional properties to a random coil and should not be thought of as representing the actual microstructure of the molecule.

At high molecular weights the random-coil pearl necklace model predicts

$$\frac{f}{f_{min}} \propto M^{\frac{1}{3}}$$

assuming chemically similar linear polymers under theta solvent conditions.[1] The relationship is found to hold for lower molecular weights (down to 10^3) than assumed in its derivation (Cowie and Bywater, 1970).

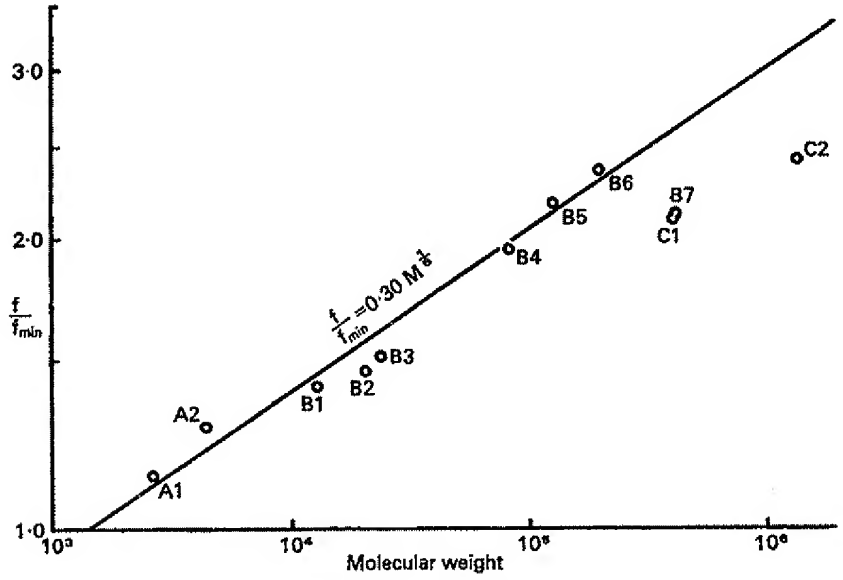

FIG. 3. Experimental relationship between the frictional ratio and molecular weight of humic acid compared to a theoretical curve for linear random coils under theta solvent conditions.

Experimentally determined values of f/f_{min} and M for the various humic acid fractions have been plotted against each other in Fig. 3. For all but the highest molecular-weight samples, log f/f_{min} increases linearly with log M, the experimental data for samples A1, A2, B1–B6 agreeing approximately with the expression

$$\frac{f}{f_{min}} = 0{\cdot}30\, M^{\frac{1}{3}}.$$

Samples B7, C1 and C2 had f/f_{min} values about 20 per cent below those necessary for a continued linear fit.

The relationship observed for the first eight points is predicted by both models (ii) and (iv). However, the hard oblate spheroids (or flat

[1] A theta solvent is one in which the forces tending to expand the molecule beyond its random flight conformation (i.e. volume exclusion effects and solvent-polymer attractions) are exactly cancelled by the forces tending to contract the molecule (polymer-polymer attraction). It is believed that such conditions may be approximated in our experiments.

sheets of uniform thickness), favoured by Piret *et al.* (1960), seem intuitively unlikely for the heterogeneous, relatively highly-charged humic acid molecules. The random-coil model is a more acceptable structure considering the chemical nature and mode of formation of humic acid. If the random coils were branched rather than linear, the above relationship would still apply provided that the mean number of branches per molecule did not vary with molecular weight.

Trends in chemical composition with molecular weight appear to be the rule for humic acid. Chemical analyses by Swift and Posner (1972) on a fractionated 20 °C NaOH extract similar to the one considered here show that although sulphur and non-amino nitrogen contents remain fairly constant at 1·5 per cent and 1·2 per cent respectively, amino nitrogen and phosphorus contents rise from about 0·8 per cent and 0·02 per cent for sample B1 to 1·6 per cent and 0·05 per cent for samples B6 and B7. Regardless of the way in which chemical structure (and, presumably, physical microstructure) varies with molecular weight, the only requirement for 'random flight' behaviour is that the 'equivalent freely jointed chains' proposed by Kuhn and described by Flory (1953) and Tanford (1961) should have 'statistical segments' of constant mass squared to root mean square length ratio.

Either non-theta conditions (due to insufficiently high ionic strength), or the suspected increase in polydispersity at higher molecular weights, would have favoured the experimental f/f_{min} varying according to a somewhat higher power of M than $\frac{1}{3}$. That this was not the case may mean that these effects were unimportant or, alternatively, that they were offset, either by an increasing mass squared to root mean square length ratio for the 'statistical segments', or by greater numbers of branched sites in large molecules. The latter possibilities may also account for the levelling of f/f_{min} values for the higher molecular-weight samples.

The random-coil model with the possibility of branching, particularly at higher molecular weights, not only accounts for the observed relationship throughout the molecular-weight range but also is an acceptable structure when the chemical nature and mode of formation of humic acid are taken into consideration.

If this theory is correct then the humic acid molecule in solution might be visualized as a series of charged, occasionally branching strands. The strands coil and wind randomly with respect to both space and time so that the mean distribution of molecular mass is spherical and Gaussian about the centre. Branching results in increased coil density within the molecule giving rise to more compact spheres than for a linear molecule of equivalent molecular weight. The structure is perfused with solvent molecules which are able to exchange with bulk-solvent molecules. At the higher molecular weights, however, the solvent, whilst flowing freely through the periphery of the sphere, would be effectively trapped in the central regions.

By applying the pearl necklace concept to random coils, Kirkwood and Riseman (1948) have shown that the molecules exhibit the frictional properties of an equivalent hydrodynamic sphere of radius R_e. At high molecular weights $R_e \rightarrow 0{\cdot}665\ R_G$ where R_G is the radius of gyration

(i.e. the root mean square average of the distance of mass units from the centre of gravity). It can be shown from the relationships given by Flory (1953) and Tanford (1961) that

$$R_G = \frac{kT}{0{\cdot}665 \times 6\pi\eta D}$$

and

$$\frac{R_G}{M^{\frac{1}{3}}} = \frac{(kT/N)^{\frac{1}{3}}}{0{\cdot}665 \times 6\pi\eta} \cdot \frac{(1-\bar{\nu}\rho)^{\frac{1}{3}}}{s^{\frac{1}{3}}D^{\frac{1}{3}}}$$

(N = Avogadro's No.)

For linear polymer species in random flight conformation (i.e. under theta solvent conditions) $R_G \propto M^{\frac{1}{2}}$ and therefore $R_G/M^{\frac{1}{2}}$ is a constant for the species. For a branched molecule R_G and $R_G/M^{\frac{1}{2}}$ will be decreased by an amount related to the degree of branching (Zimm and Stockmayer, 1949).

The values of R_G and $R_G/M^{\frac{1}{2}}$ derived from the experimental data (Table 1) deviate from linearity at high molecular weights in the same way that the $f/f_{\min}$ values did. The value of $R_G/M^{\frac{1}{2}}$ was approximately constant for the first eight fractions (mean $= 2{\cdot}9 \times 10^{-9}$ cm). This parameter allows simple calculation of the approximate diameter of humic acid molecules of a given molecular weight. Some idea of molecular size is given by doubling the R_G values in Table 1.

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